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(54) Title: NOVEL COMPONENT OF AMYLOID IN ALZHEIMER'S DISEASE AND METHODS FOR USE OF SAME

(57) Abstract

A gene, NACP, is disclosed along with its nucleotide and amino acid sequence. Also disclosed are diagnostic and therapeutic methods of utilizing the NACP nucleotide and polypeptide sequences. The NACP polypeptide is a precursor of NAC, a peptide associated with amyloid deposits in the brains of patients with typical neuropathological features of Alzheimer's disease (AD). Also disclosed is the amino acid sequence of NAC and of two contiguous fragments thereof, X and Y peptides. Diagnostic and therapeutic methods relating to amyloid disorders associated with NAC are also disclosed.

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NOVEL COMPONENT OF AMYLOID IN

ALZHEIMER'S DISEASE AND METHODS FOR USE OF SAME

STATEMENT OF GOVERNMENT SUPPORT

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RELATED U.S. PATENT APPLICATIONS

This application is a continuation in part of U.S. patent application serial no.

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BACKGROUND OF THE INVENTION

10   *Field of the Invention*

This invention relates to diagnosis and treatment of neuronal abnormalities, in

particular the deposition of amyloid plaques characteristic of Alzheimer's

Disease.

Description of Related Art

15   The most common cause of disabling dementia in humans is Alzheimer's

disease ("AD"). Its incidence increases sharply with age, and it is a major

public health problem in our aging population. Persons suffering from

Alzheimer's disease show a characteristic neuropathology, including synaptic

loss, senile plaques and neurofibrillary tangles. Neurofibrillary tangles comprise

20   paired helical filaments ("PHF") (D.L. Selkoe, *et al.*, *Science*, 235:873-876,

1987). A senile plaque commonly comprises a mass of disorganized neurites surrounding a deposit of extracellular filaments of an amyloid polypeptide called A4 or β amyloid protein ("Aβ").

5 Deposition of fibrillar deposits of Aβ a 39/43 residue amyloid, is considered the pathological hallmark of AD. Recently, molecular cloning based on the sequence of the Aβ protein indicated that it is encoded as part of a larger precursor (PreA4) that maps to chromosome 21 (Kang, et al., Nature, 325:733-736, 1987; Goldgaher, et al., Science, 235:877-880, 1987; Tanzi, et al., Science, 235:880-884, 1987; Robakis, et al., Proc. Natl. Acad. Sci. 84:4190-

10 4194, 1987). There are three major alternatively spliced products of the amyloid mRNA (Ponte, et al., Nature, 331:525-527, 1988,; Tanzi, et al., Nature, 331:528-530, 1988; Kitaguchi, et al., Nature, 331:530-532, 1988). The smallest of these products, the 695-residue precursor protein (PreA4$_{695}$), has been synthesized in vitro and shown to be a N-glycan membrane protein that spans

15 the lipid bilayer once (Dyrks, et al., EMBO J., 7:949-957, 1988). Two other forms of PreA4 (PreA4 751 and PreA4 770) contain a 56 residue insert which has a protease-inhibitory function. The amyloidogenic A4 protein is derived in part from the transmembrane domain and from part of the adjacent extracellular domain. A precursor-product relationship has been demonstrated.

20 The A4 gene is expressed in brain and peripheral tissues, such as muscle and epithelial cells (Goeder, EMBO J., 6:3627-3632, 1987; Bahmanyar, et al., Science, 237:77-88, 1987; Zimmerman, et al., EMBO J., 7:1365-1370, 1988; Shivers, et al., EMBO J., 7:1365-1370, 1988), yet for reasons still unknown, the amyloid deposits in AD are confined to the brain.

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Recently, *in situ* hybridization analyses were published that indicate an alteration of the amount of PreA4 mRNA in brains of AD patients when compared to normal individuals (Higgins, *et al.*, *Proc. Nat'l Acad. Sci. USA*, 85:1297-1301, 1988; Cohen, *et al.*, *Science*, 237:77-88, 1987; Lewis, *et al.*, *Proc. Nat'l Acad. Sci. USA*, 85:1691-169, 1988). These results implicate a role for gene regulation in AD.

In addition to Aβ, heparan sulfate proteoglycan, ferritin, immunoglobulins, and many acute phase proteins such as α-1 antichymotrypsin, apolipoprotein E, complements, serum amyloid P, and trace peptides have been reported to be associated with amyloid. However, supportive biochemical data demonstrating the presence of these proteins in amyloid preparations from the brains of Alzheimer's victims are not yet available, raising the possibility that these may not be intrinsic components of amyloid.

All forms of amyloid in amyloid deposits, including the Aβ, show a significant β-pleated sheet component (Snow, A.D., *et al.*, 1987). Yet the precursor of amyloid Aβ protein is soluble and does not exhibit a significant β-pleated sheet component. Recent studies of C. Haass, *et al.*, *Nature*, 359:322-325, (1992); P. Seubert, *et al.*, *Nature*, 359:325-327 (1992); M. Shoji, *et al.*, *Science*, 258:126-129 (1992), have demonstrated that Aβ is generated and secreted from various types of cells under physiological conditions, implying that Aβ is soluble in aqueous solutions.

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The physiological process responsible for changing the structure of the precursor protein is the subject of much enquiry. Recently a study by Roses, *et al.*, demonstrates that apolipoprotein E binds Aβ (*Proc. Natl. Acad. Sci. USA*, 90:1977-1981, 1993). Thus, apolipoprotein E may act as a molecular

5   chaperone that mediates the β-pleated amyloid formation of Aβ as suggested by T. Wisniewski, *et al.* (*Neurosci. Lett.*, 135:235-238, 1992).

New and further information concerning the molecular biology involved in formation of amyloid deposits such as those found in Alzheimer's disease awaits discovery of additional intrinsic constituents associated with amyloid in

10   the brains of those affected with Alzheimer's disease. On a physiological basis, recent studies have shown that amyloid deposition might be the result of aberrant processing of APP, and its abundance is an important parameter to consider in diagnosing the disease on a neuropathological basis. In addition, the cognitive dysfunction that characterizes AD is apparently attributable to

15   synaptic loss (Terry, *et al.*, *Ann.Neurol.*, 30:572-580 (1993); Mattson, *et al.*, *TINS*, 16:409-414 (1993)). Recent studies strongly suggetsthat there is a connection between the abnormal processing of synaptic proteins and amyloid formation (Masliah, *et al.*, *Brain Path.*, 3:77-85 (1993)).

However, despite the knowledge that AD is related to neuritic plaques and

20   synaptic loss, diagnosis of the disease is difficult. Currently, the only way of confirming the presence of these lesions in a living patient is by brain biopsy. However, this technique is rarely utilized because of the substantial risks to the patient involved in performing it. As a result, AD is usually diagnosed on the basis of clinical symptoms and the results of neuropsychological tests.

25   Nonetheless, because AD can be mimicked by other disorders (such as

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depression), confirmation of an AD diagnosis often cannot be conclusively made until autopsy.

Several methods for *in vivo* diagnosis of AD have been proposed but have not yet yielded definitive results. One such approach attempts to detect amyloid
5   and/or the precursor protein for it (APP) in blood and cerebrospinal fluid. These measurements have not, however, been shown to positively correlate to the development of neuritic plaques in AD. Another approach involves detection of a mutated form of the gene for the amyloid precursor protein. While the presence of this genetic alteration appears to be more predictive of
10   AD than does circulating levels of amyloid and APP, the mutated gene is only found in some familial cases of AD. As a result, presence of the mutated gene would correlate to the onset of AD in less than 1% of all potential AD cases.

In vivo diagnosis of AD is further limited by the blood/brain barrier. Because of the barrier, detection of amyloid deposits by binding assays (and evaluation
15   of synaptic loss associated with dementia) has been limited to autopsy studies (see, e.g., Masliah, *et al., Am. J. Pathol.*, 137:1293-1297, 1990 [quantitation of synapse loss in brain tissue section through use of labelled anti-synaptophysin antibodies]).

Further, the blood/brain barrier has also prevented (to date) the effective use
20   of antibodies for *in vivo* diagnosis and therapy of AD. Thus, a promising *in vitro* use of a monoclonal antibody 10H3 which targets amyloid deposits (Maj- ocha, *et al. J. Nucl. Med.*, 33:2184-2189) has not yet been extended to an *in vivo* application. Due to the size of antibodies like 10H3, there is some doubt whether they can successfully and innocuously cross the blood/brain barrier.

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Clearly, a need exists for a noninvasive method for *in vivo* detection of amyloid deposits in brain tissue of patients who are suspected of having AD. In combination with present techniques for clinical diagnosis of AD, such a technique would be useful in confirming a diagnosis of, and evaluating the prognosis for, the disease.

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SUMMARY OF THE INVENTION

Novel peptides obtained by purification and analysis of the amino acid sequences in an amyloid preparation of brain tissue obtained from patients with symptoms of Alzheimer's disease have been shown to be fragments of a novel
5   amyloid component ("NAC"). Antibodies were raised against synthetically produced fragments of these novel peptides and used in immunohistochemical and electron microscopic analyses demonstrating that the peptides are localized in amyloid fibrils in AD brain tissue and are amyloidogenic.

Complementary DNA ("cDNA") encoding a 140 amino acid protein identified as
10   the precursor ("NACP") of NAC is provided. NACP is a highly abundant synaptic protein, which degrades to form NAC. NAC is self-aggregating; i.e., it has a significant ability to bind to itself and become part of amyloid fibrils and neuritic plaque. The invention therefore provides NAC and NACP peptides useful as ligands to identify and quantify syanpses and plaques toward
15   diagnosis and monitoring of diseases associated with synaptic loss and neuritic plaque formation, such as AD. The invention also provides methods for treatment of such diseases.

In particular, in the preferred diagnostic embodiment of the invention, a detectably labelled NAC/NACP peptide which will specifically bind NAC
20   deposits in brain tissue, is administered parenterally to a mammal (preferably a human). Binding of the administered peptide to NAC/NACP in brain tissue is detected using suitable *in vivo* diagnostic imaging techniques. Most preferably, this detection will be by positron emission tomography (PET) or single photon emission computed tomography (SPECT).

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In another aspect of the invention, the binding data generated as described above is evaluated with any clinical signs of a neuropsychological disorder to assist in confirmation or refutation of an initial diagnosis of AD. To the extent that the binding data reveals the extent of amyloid deposition, the data may

5   also be used to evaluate the prognosis for a patient with a confirmed diagnosis of AD. Thus, the diagnostic method of the invention will provide physicians with valuable information concerning the medical status of a patient who is suspected of suffering from AD.

In another aspect of the invention, the binding data described above is

10   evaluated with the results of tests for synapse loss in brain tissue to assist in confirmation of an AD diagnosis and to evaluate the prognosis for the patient. Data evidencing a relationship between amyloid deposition and synapse loss in AD brain tissue will also be of use in research toward understanding the etiology of AD.

15   In another aspect of the invention, labelled NAC/NACP peptides are utilized in in vitro studies of amyloid deposition in sections of brain tissue for use in confirming an AD diagnosis and/or for research purposes. For example, in vitro (and in vivo) use of detectably labelled NAC/NACP peptides may be used to evaluate agents to inhibit NAC formation, binding and deposition.

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BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 A shows immunohistochemical staining with anti-NAC antibodies of amyloid in diffuse, primitive, and mature plaques on slides of brain sections from patients with symptoms of Alzheimer's disease. In Panel A hippocampal

5   sections were stained with anti-NAC antibody, anti-X1. In Panel B, in addition to amyloid staining, occasional staining of dystrophic neurites (arrows) was detected with a anti-NAC antibody, anti-Y. Absorption with the corresponding peptide   eliminated   the   staining.   Panel   C   shows   absence   of immunohistochemical staining by anti-sera to NAC on slides of AD brain

10   sections when pre-absorbed with NAC peptides. Panel D shows an electron micrograph of specific staining by anti-X1 antibody on amyloid fibrils (arrows) in AD brain sections. Amyloid fibrils were also stained with anti-Y antibody.

Figure 2 A shows the nucleotide sequence of cDNA encoding the precursor of the NAC protein and the 140 amino acid sequence of the NAC precursor

15   protein encoded by a 420 bp open reading frame with the X and Y fragments located contiguously in the middle of the precursor. The nearest in-frame stop codon (TAA) upstream to the putative initiation methionine codon is marked by an asterisk. The termination codon is marked by two asterisks. Sequence for X and Y peptides are boxed. Synthetic oligonucleotide mixtures used for PCR

20   are indicated as lines above the corresponding cDNA. Polyadenylation signals are underlined.

Figure 2 B is a graph showing the hydropathy profile of the NAC precursor protein with the NAC region being the most hydrophobic.

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Figure 3 is a Western blot of brain homogenate with anti-NAC polyclonal antibody anti-X1. NAC precursor protein in lane 4 is detected as a M, 19K protein. The bacterially expressed NAC precursor protein expressed in E. coli transfected with pSENACP migrated in lanes 2 and 5. Lanes 1 and 6 contain

5   E. coli transfected with pSE380 vector control; and lanes 3 and 4 show normal human brain. Lanes marked with (+) indicate X1 antibody was preabsorbed with X1 peptide; while those marked (-) indicated X1 antibody was preabsorbed with a control peptide. The arrow indicates NACP detected as a M, 19K protein whose staining was blocked by preabsorption of the antibody

10   with X1 peptide, thus showing specificity of the antibody.

Figure 4 A is a sequence listing of seven repeated sequence motifs in the NAC precursor amino acid sequence.

Figure 4 B is a sequence listing showing homology in the NAC precursor amino acid sequence at amino acids 48-56 and 70-78. Bold letters indicate the

15   common amino acids among the repeat.

Figure 4 C is a comparison of the cDNA listings of EST01420 (EMBL/GenBank Libraries) and the NAC precursor showing homology therebetween at the N-terminal region of NAC.

Figure 5 is a Northern Blot of mRNA for NACP. Lane 1 shows normal adult

20   midfrontal cortex (female, aged 88); lane 2 shows cerebellum from the same individual as lane 1; lane 3 shows fetal whole brain (female, 24 week fetus); lane 4 shows midfrontal cortex from individual with AD (female, aged 83); lane 5 shows cerebellum from the same individual as Lane 4; lane 6 shows normal adult liver (male, aged 18); lane 7 shows normal child lung (male, aged 7).

Figure 6 A depicts self-aggregation of NAC peptide at various times and peptide concentrations (10-300 μM) as measured by turbidity at OD 400 nm. The NAC aggregation was studied in PBS (pH 7.4) solution at 37°C.

5   Figure 6 B depicts self-aggregation of NAC peptide (concentration, 300 μM) at various times and temperatures (4°, 22°, and 37°C) as measured by turbidity at OD 400 nm. The NAC aggregation was studied in PBS (pH 7.4) solution.

Figure 7 is a Western blot analysis of NAC peptide aggregation. A NAC peptide monomer migrated at an apparent molecular mass of the 3500 Da.
10   The signal intensity of 3500 Da band was significantly decreased on Days 5 and 7. On the other hand, aggregated NAC peptide was found at the top of the gel. This signal increased to a maximum by Day 3. No intermediate-size bands were observed.

Figures 8A and B show birefringence of Congo red-stained NAC peptide
15   viewed by cross-polarization microscopy. Bright-field (A) and cross-polarized light (B) pictures of NAC peptide preparation stained with Congo red are shown.

Figure 9 is an electron micrograph of aggregated NAC peptide.

Figures 10 A through D show (both macro- and microscopically)
20   immunostaining of NACP in rat brain using anti-NACP(131-140) with streptavidin-biotin-peroxidase (SAB) method. In FIGURE 10 A is a macroscopic image of stained sagittal brain section showing that NACP immunoreactivity was relatively strong in the neocortex, olfactory region, caudoputamen,

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hippocampus, and cerebellar cortex compared to the thalamus and brain stem. Higher magnification microscopic analysis showed a characteristic granular immunoreactivity throughout the brain. In FIGURE 10 B is a microscopic image of stained cerebellar cortex. In FIGURE 10 C is a microscopic image of stained

5   hippocampal dentate gyrus. In FIGURE 10 D is a microscopic image of stained cerebral cortex.

Figures 11 A through I are photographs obtained by laser scanning confocal microscopy of sections double-labeled for SY38 (mouse monoclonal anti-synaptophysin antibody; labeled with FITC and shown by bright patches in

10   panels A, D and G) and NACP(131-140/SEQ.ID.No.6; shown by bright patches in panels B, E and H). The right hand panels (C, F and I) correspond to the electronically merged image; colocalization of NACP with synaptophysin is indicated by bright patches. Panels A-C are derived from staining of neocortex tissue; panels D-F are derived from staining of glomeruli of the olfactory bulb;

15   and, panels G-I are derived from staining of the cerebellar cortex. The scale bar (a horizontal white line across the lower right hand corner of panel A) is equal to 15 μm.

Figure 12 is a bar graph developed by computer-aided quantification of colocalization of NACP with synaptophysin in the presynaptic terminals of rat

20   brain tissue. In the cortical regions a large percentage of the synpatophysin-immunoreactive terminals contained NACP. In contrast, in subcortical regions, a lower proportion of the synapatophysin-immunoalabeled axosomatic nerve terminals contained NACP.

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Figure 13 is a photograph obtained by immunoelectron microscopy which reveals the synaptic vesicle membrane localization of NACP. Vibratome sections were immunostained with anti-NACP(131-140) and analyzed by electron microscopy.

5   Figure 14 is a Western blot quantifying NACP, APP, and synaptophysin in rat brain sections. Immunostaining of NACP, APP, and synaptophysin bands was carried out using anti-NACP(131-140), mouse monoclonal antibody, 22C11, and mouse monoclonal antibody, SY38, respectively. Signal intensity was quantified by scanning by a densitometer. Each value is shown as relative amount of

10   protein normalized to the value in frontal cortex. NACP is highly concentrated in olfactory bulb, frontal cortex, striatum, and hippocampus , whereas APP and synaptophysin are distributed uniformly throughout the brain.

Figure 15 A-B shows, in bar graph form, the number of NACP containing and synaptophysin containing synaptic terminals present per 100 sq/μm of human

15   frontal cortex brain tissue.

Figure 15 C-D shows, in bar graph form, the pixel intensity detected per synapse of human frontal cortex brain tissue indicative of the average quantities of NACP and synaptophysin contained in each synapse. The solid bars are indicative of the values obtained in brain tissue from persons without AD; the

20   slashed bars are indicative of the values obtained in brain tissue from persons suffering from AD.

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Figure 16 shows immunolabeling of NACP and synaptophysin in human brain tissue obtained from a healthy person (upper panels) and from a person suffering from AD (middle panels). The left and right images from the middle panel are electronically merged in the lower panel. Areas showing the brightest in the lower panel indicate colocalization of NACP and synaptophysin.

Figure 17 shows immunolabeling of NACP and synaptophysin in mature plaques (the 2 upper left hand panels) and in diffuse plaques (the 2 upper right hand panels). The left and right images from the upper panels are electronically merged in the lower panel. Areas showing the brightest in the lower panel indicate colocalization of NACP and synaptophysin.

Figure 18 shows immunolabeling of β-amyloid (left hand panels) and NAC (right hand panels, with results electronically superimposed on the left hand panels) in brain tissue from healthy, elderly persons (panels A and B), from persons suffering from the early stages of AD (panels C and D), and from persons suffering from advanced stages of AD.

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DETAILED DESCRIPTION OF THE INVENTION

A.   FUNCTIONAL AND STRUCTURAL CHARACTERISTICS OF NAC AND
     NACP.

The present invention provides a novel amyloid component (NAC). As used
herein "NAC" shall mean Non-Aβ component of AD amyloid. "Aβ" as used
herein shall mean fibrillar deposits of the A4 protein, a 39/43 residue amyloid.
"AD" as used herein shall mean Alzheimer's Disease. "NAC associated amyloid
disorder" shall refer to diseases associated with the excessive formation of
amyloid in brain tissue, concommitant synaptic loss, and related cognitive
dysfunction. This novel component of amyloid was discovered by analysis of
the entire amino-acid sequences in an amyloid preparation of the frontal cortex
of patients with typical neuropathological features of Alzheimer's disease (AD)
using methods of purification in SDS and sequencing well known in the art.
Hence, NAC is the second intrinsic component after Aβ to be found in AD
amyloid.

NAC, which is expressed as a larger precursor polypeptide NACP, was found
by both biochemical and immunohistochemical evidence to be an intrinsic
component of amyloid in AD brain tissue. Copurification of NAC with amyloid
in the presence of SDS and immunological localization on amyloid fibrils at the
electron microscopic level shows that NAC is localized in neuritic plaques and
amyloid fibrils.

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As shown in FIGURE 2, NACP is encoded by a 1560 base pair polynucleotide (SEQ. I.D. NO. 1) with a 420 bp open reading frame which encodes a 140 amino acid polypeptide NACP (SEQ. I.D. NO. 2) that is the precursor of NAC, a polypeptide of at least 35 amino acids in length (SEQ. I.D. NO. 3). *In vivo*,
5   NACP is recovered in the cytosolic fraction of human brain homogenate as a protein with an apparent molecular mass of 19,000 Da. NACP has seven repeated KTKEGV amino acid motifs, but no signal petide sequence nor N-linked glyclosylation sites. NAC is located in the most hydrophobic portion of NACP. NAC is at least 35 amino acids and has a molecular weight of
10   approximately Mr 3,500. Within NAC, two new amyloid sequences have been identified and known herein as the "X and Y peptides", which are encoded contiguously in the most hydrophobic domain (SEQ. I.D. NOS. 4 and 5, respectively). The definite length of NAC was not determined due to the use of enzymatic digestion in its preparation.

15   The association of NAC in amyloid deposits in AD brain tissue differs from that of both Aβ and α1-antichimotrypsin (ATC), two proteins generally used as indicators of the presence of amyloid in Alzheimer's Disease. Recent work has shown that 50% of intracellular neurofibrillary tangles (NFT) and 100% of extracellular NFT contain Aβ (G. Perry, *et al.*, *Am. J. Pathol.*, 140:283-290,
20   1992). Immunohistochemical studies of the distribution of NAC in AD brain tissue (See Example 2 herein) found that NAC was not present in NFTs.

The association of NAC with amyloid in the brains of patients with the symptoms of Alzheimer's Disease (AD) is high. Although ATC has been reported to be localized on amyloid fibrils in brain tissue at the electron
25   microscopic level (C. R. Abraham, *et al.*, *Cell*, 52:487-501, 1988), biochemical analysis of amyloid AD brain tissue prepared and analyzed as in Examples 1-3

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below, revealed no ATC. This result suggests that the amount of ATC in amyloid may be too small to detect in the present preparation, or, alternatively, that the association of ATC with amyloid may be less significant than that of NAC and, therefore, ATC may be lost during preparation of amyloid used

5   herein.

Thus, as an indicator of the deposition of amyloid in brain tissue, NAC is both more specific to neuritic plaque and amyloid fibrils than ATC and less likely to be lost in preparation of tissues to be tested than ATC.

NAC is strongly hydrophobic and has characteristics associated with a

10   tendency to form a β-pleated secondary protein structure. When synthesized chemically, NAC aggregates and precipitates easily in aqueous solution in a time, concentration and temperature-dependent manner. More particularly, synthetic NAC was detected initially as a monomer of 3500 Da, but became aggregated in aqeuous solution into a higher molecular weight molecule that

15   could not migrate into an electrophoretic gel. On Congo red staining, the NAC aggregate showed green-gold birefringence when viewed with a poloarized light microscope and had a fiber-like structure when viewed through an electron microscope.

Based on the relative yield of peptides X, Y and Aβ sequences in amyloid

20   preparations, the concentration of NAC in amyloid seems to be less than ten percent that of Aβ. Further, double-immunostaining of NAC with β-amyloid antibodies revealed that NAC is more abundant in mature than in diffuse plaques. Interestingly, diffuse plaques from "normal" control tissue do not react with anti-NAC, whereas early and advanced AD cases cotaining large numbers

25   of diffuse and/or at least some mature plaques display relatively strong anti-

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NAC reactivity (in approximately 30-50% of the plaques) (see, FIGURE 18).
These data suggest that (1) there is a connection between metabolism of
presynatpic proteins and plaque formation, and (2) NAC follows diffuse β-
amyloid accumulation into mature plaques.

5   In contrast, it does not appear that NACP is substantially present, if at all, in
amyloid. For example, no other sequences of NACP besides the X and Y
peptides were detected in the peaks eluted from HPLC analysis of the NACP
protein.   Further, while NAC was identifed in immunostained amyloid on
Western or dot blot, NACP was not. Thus, it appears that NAC can form
10   amyloid *in vivo* after cleavage from its precursor (NACP) and is likely to play
a substantial role in amyloidosis. However, because the amyloid found in the
brain tissue of humans with confirmed diagnoses of AD differs in structure from
NAC aggregates alone, it is likely that NAC is not the sole component of
amyloid. Rather, it is most probable that NAC is involved in the initial stages
15   of amyloid formation, leaving the principal development of amyloidosis to the
accumulation of β-amyloid.

It should be appreciated, however, that observations have been made that
proteins that bind to β-amyloid retard its accumulation (see, e.g., Strittmatter,
et al., Proc.Natl.Acad.Sci.   USA, 90:8098-8102 (1993); Fraser, *et al.,*
20   *J.Neurochem.,* 61:298-305 (1993); and, Schwarzman, *et al., Ann.N.Y.Acad.Sci.,*
6:139-143 (1993)). Thus, with the knowledge of NAC's role in amyloidosis set
forth herein, it can be reasonably expected that binding of NAC by NAC
polypeptides will retard its accumulation as well, thereby slowing the
progression of disease associated with amyloid plaque formation.

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With respect to NACP, immunostaining indicates that it, like the APP precursor of β-amyloid, is a presynaptic protein. Specifically, using the streptavidin-biotin-peroxidase staining method, rat brain sections were immunostained by an antibody raised to the NACP amino acid sequence from position 131-140 (see,

5   SEQ.ID.No.2) and position 1-9 (*id*.). Throughout the various cortical and subcortical areas of the rat brain, anti-NACP (131-140) and (1-9) immunostained the neuropil in a characteristic punctate pattern. Neuronal cell bodies, glial cells and blood vessels were not immunostained.

NACP does not have a signal sequence, which suggests that NACP proteins

10   remain localized in neuronal cytoplasm where NACP is expressed. However, it has been discovered that NAC can seep out of cells under certain conditions, such as serum deprivation. Further, as shown in FIGURE 4A, the NACP protein is characterized by repetitive motifs. The KTKEGV motif is repeated seven times, but the amino acid positions 2 to 6 are sometimes substituted.

15   In addition, as shown in FIGURE 4B, amino acids 48-56 and 70-78 of the NACP protein are homologous. These repeated motifs can prove useful in determining the secondary and tertiary protein structure as well as the biological function and metabolism of this protein.

For instance, in accordance with the teachings of P. J. Kennelly, *et al.* (*J. Biol.*

20   *Chem.*, 256:15555-15558, 1991), the threonine residues in the KTKEGV motif would offer favorable targets for protein kinase C (PKC). The action of this enzyme is known to be critical in determining the functional state of neurons (Y. Nishizuka, *Nature*, 334:661-665, 1988).

In addition, laser scanning confocal microscopic analysis of sections double

25   immunolabeled with antibodies against NACP and synaptophysin (a synaptic

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vesicle protein; see, e.g., Masliah, et al., J.Neurosci., 11:2759-2767 (1991))
showed that both markers colocalized in the great majority of the presynaptic
terminals, and that NACP is colocalized with synaptophysin in approximately
80% of the presynaptic boutons and in the neuritic component of plaque

5   (FIGURES 11 through 17). Ultrastructural analysis of sections immunolabeled
with NACP confirmed the synpatic localization of this protien and showned that
NACP was associated with the synaptic vesicles (FIGURE 13). As compared
to synaptophysin and APP (which are distributed fairly evenly throughout the
brain), NACP was concentrated in the telecephalon, suggesting a functional

10   role for NACP in that region of the brain (FIGURE 14).

As shown in FIGURE 3, NACP is detected in the cytosolic fraction of brain
homogenates and comigrates on Western blots with NACP synthesized in E.
coli from NACP cDNA. NACP was not detected in a particulate fraction from
human cortex or from NACP-expressing E. coli. NACP mRNA is expressed

15   principally in the brain, but is also expressed in low concentration in all tissues
examined except in liver, suggesting that it has ubiquitous functions as well as
brain specific functions.

Interestingly, in AD brain, the total population of NACP-containing presynaptic
terminals is significantly diminished (by 30-40% see, FIGURE 15) as compared

20   to "normal" brain tissue; i.e., brain tissue without a diagnostically significant
quantity of plaque (defined further below). At the same time, although the
total population of such terminals is decreased in the AD brain, the
concentration of NACP in each remaining presynaptic bouton, indicating a
mechanism to compensate for the overall level of NACP-expressing terminals.

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As further evidence of NACP as a synaptic protein, search of the
EMBL/GenBank DNA sequence databases reveals homologies between NACP
and rat brain synucleins, the electric organ synpase of *Torpedo califomica*
(Pacific electric ray) and to bovine phosphoneuroprotein 14, a brain specific
5   protein present in synapses around neurons but not in glial cells and Purkinje
cell bodies. This group of small, acidic, brain-specific proteins have common
repetitive sequence motifs and similar hydrophobic profiles (see, Maroteaux,
et al., Mol.Brain Res., 11:335-343 (1991); Maroteaux, *et al., J.Neurosci.,* 8:2804-
2815 (1988); and, Nakajo, *et al., Eur.J.Biochem.,* 217:1057-1063 (1993)).

10   In addition, according to the GenBank database, homology exists between
NACP and EST01420, a human 223 bp sequence recently identified by random
sequencing of human brain cDNA (M. D. Adams, *et al., Nature,* 355:632-634,
1992). Comparison of the sequence of these proteins expressed in the human
brain, as shown in FIGURE 4C, indicates the two proteins are substantially
15   homologous in the N-terminal region, but the EST01420 sequence has the
termination codon at base pair position 206, and, therefore, could encode only
51 amino acids.

NACP therefore appears to be a member of a family of synaptic proteins
having hydrophobic regions centered in an otherwise hydrophilic molecule.

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B.   NAC/NACP POLYNUCLEOTIDES AND POLYPEPTIDES.

The term "substantially pure" means any NAC or NACP polypeptide of the
present invention, or any gene encoding a NAC or NACP polypeptide, which
is essentially free of other polypeptides or genes, respectively, or of other

5   contaminants with which it might normally be found in nature, and as such
exists in a form not found in nature.

As used herein, the term "functional polypeptide" refers to a polypeptide which
possesses a biological function or activity that is identified through a defined
functional assay and which is associated with a particular biologic, morphologic

10   or phenotypic alteration in the cell. The biological function can vary from a
polypeptide fragment as small as an epitope to which an antibody molecule
can bind to as large as a polypeptide which is capable of participating in the
characteristic induction or programming of phenotypic changes within a cell.
A "functional polynucleotide" denotes a polynucleotide which encodes a

15   functional polypeptide as described herein.

For example, preferred NAC/NACP polypeptides of the invention will be those
which will effectively cross the blood/brain barrier without toxic effect. NAC
polypeptides of the invention will specifically bind NAC in vivo; the peptides will,
therefore, have at least one binding site for NAC.

20   Further, the NAC/NACP polypeptides should not be pathogenic or
immunogenic. To the former end, the peptides are soluble and, in the case of
NAC peptides, will reversibly bind NAC. To the latter end, the polypeptides are
preferably purified from a human or will be synthesized. "Synthesized" in this
context refers to peptides produced through human intervention, whether by

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chemical synthesis, recombinant genetic techniques or modification of an isolated native peptide.

It should be noted that NAC/NACP polypeptides used in the method of the invention may differ in amino acid sequence or structure but still retain the

5   same biological activity as described above. Such modifications may be deliberately made (by, for example, site-directed mutagenesis) or may occur spontaneously. In either case, the invention will encompass the use of NAC/NACP peptides which have the same phenotype regardless of differences in structure and length between the peptides. These phenotypically similar

10   peptides will be considered to "substantially similar" to one another.

On the molecular level, a molecule is said to be "substantially similar" to another molecule if the sequence of amino acids in both molecules is substantially the same. Substantially similar amino acid molecules will possess a similar biological activity. Thus, provided that two molecules possess a

15   similar activity, they are considered variants as that term is used herein even if one of the molecules contains additional amino acid residues not found in the other, or if the sequence of amino acid residues is not identical. As used herein, a molecule is said to be a "chemical derivative" of another molecule when it contains additional chemical moieties not normally a part of the

20   molecule. Such moieties may improve the molecule's solubility, absorption, biological half life, etc. The moieties may alternatively decrease the toxicity of the molecule, eliminate or attenuate any undesirable side effect of the molecule, etc. Moieties capable of mediating such effects are disclosed, for example, in *Remington's Pharmaceutical Sciences*, 16th ed., Mack Publishing Co., Easton,

25   Penn. (1980).

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Minor modifications of the NAC primary amino acid sequence may result in proteins which have substantially equivalent activity as compared to the NAC polypeptide described herein. Such modifications may be deliberate, as by site-directed mutagenesis, or may be spontaneous. All of the polypeptides

5   produced by these modifications are included herein as long as the biological activity of NAC still exists. Further, deletion of one or more amino acids can also result in a modification of the structure of the resultant molecule without significantly altering its biological activity. This can lead to the development of a smaller active molecule which would have broader utility. For example, one

10 can remove amino or carboxy terminal amino acids which may not be required for NAC biological activity.

The term "conservative variation" as used herein denotes the replacement of an amino acid residue by another, biologically similar residue. Examples of conservative variations include the substitution of one hydrophobic residue

15 such as isoleucine, valine, leucine or methionine for another, or the substitution of one polar residue for another, such as the substitution of arginine for lysine, glutamic for aspartic acids, or glutamine for asparagine, and the like. The term "conservative variation" also includes the use of a substituted amino acid in place of an unsubstituted parent amino acid provided that antibodies raised to

20 the substituted polypeptide also immunoreact with the unsubstituted polypeptide.

By "functional derivative" is meant the "fragments," "variants," "analogues," or "chemical derivatives" of a molecule. A "fragment" of a molecule, such as any of the DNA sequences of the present invention, includes any nucleotide subset

25 of the molecule. A "variant" of such molecule refers to a naturally occurring molecule substantially similar to either the entire molecule, or a fragment

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thereof.   An "analog" of a molecule refers to a non-natural molecule substantially similar to either the entire molecule or a fragment thereof.

Similarly, a "functional derivative" of a gene encoding NACP polypeptide of the present invention includes "fragments", "variants", or "analogues" of the gene,
5   including degenerate polynucleotides whose sequence may be determined readily by one of ordinary skill in the art, which encode a molecule possessing similar activity to a NAC peptide or fragment thereof.

Thus, as used herein, NAC or NACP polypeptide and NAC or NACP polynucleotide, include any functional derivative, fragments, variants, analogues,
10   chemical derivatives which · may be substantially similar to the NAC polypeptides and polynucleotides described herein and which possess similar activity.

Peptides of the invention can be synthesized by the well known solid phase peptide synthesis methods described Merrifield, *J. Am. Chem. Soc.*, <u>85</u>:2149,
15   (1962), and Stewart and Young, *Solid Phase Peptides Synthesis*, (Freeman, San Francisco, 1969, pp.27-62), using a copoly(styrene-divinylbenzene) containing 0.1-1.0 mMol amines/g polymer.   On completion of chemical synthesis, the peptides can be deprotected and cleaved from the polymer by treatment with liquid HF-10% anisole for about 1/4-1 hours at 0°C.   After
20   evaporation of the reagents, the peptides are extracted from the polymer with 1% acetic acid solution which is then lyophilized to yield the crude material. This can normally be purified by such techniques as gel filtration on Sephadex G-15 using 5% acetic acid as a solvent. Lyophilization of appropriate fractions of the column will yield the homogeneous peptide or peptide derivatives, which
25   can then be characterized by such standard techniques as amino acid

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analysis, thin layer chromatography, high performance liquid chromatography, ultraviolet absorption spectroscopy, molar rotation, solubility, and quantitated by the solid phase Edman degradation.

As used herein, the terms "polynucleotide" or "NACP polynucleotide" denotes DNA, cDNA and RNA which encode NACP polypeptide as well as untranslated sequences which flank the structural gene encoding NACP. It is understood that all polynucleotides encoding all or a portion of NACP polypeptide of the invention, such as the NAC polypeptide(s) are also included herein, as long as the encoded polypeptide exhibits the activity or function of NACP or the tissue expression pattern characteristic of NACP. Such polynucleotides include naturally occurring forms, such as allelic variants, and intentionally manipulated forms, for example, mutagenized polynucleotides, as well as artificially synthesized polynucleotides. Such mutagenized polynucleotides can be produced, for example, by subjecting NAC or NACP polynucleotide to site-directed mutagenesis.

As described above, in another embodiment, a polynucleotide of the invention also includes in addition to NACP and/or NAC coding regions, those nucleotides which flank the coding region of the NACP structural gene. For example, a polynucleotide of the invention includes 5' regulatory nucleotide sequences and 3' untranslated sequences associated with the NACP structural gene.

The polynucleotide sequence for NACP also includes antisense sequences. The polynucleotides of the invention also include sequences that are degenerate as a result of the genetic code. There are 20 natural amino acids, most of which are specified by more than one codon. Therefore, as long as

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the amino acid sequence of NACP results in a functional polypeptide (at least,
in the case of the sense polynucleotide strand), all degenerate nucleotide
sequences are included in the invention. Where the antisense polynucleotide
is concerned, the invention embraces all antisense polynucleotides capable of
inhibiting production of NACP polypeptide.

The preferred NACP cDNA clone of the invention is defined by a sequence of
1560 basepairs, in accordance with the transcript of 1.6 kb. A minor transcript
of 3.6 kb is also found. The sequence surrounding the predicted initiator
methionine codon (GCCATGG) agrees with the Kozak consensus sequence
according to K. Kozak (*Nucleic Acids Res.*, 15:8125-8148, 1987). The nearest
in-frame stop codon is found 18 bp upstream of the ATG initiation codon. As
shown in FIGURE 2A, the nucleotide sequences encoding the X- and Y-
peptide sequences are localized contiguously in the middle of the precursor
peptide at bp 233 to 337. The preferred NACP cDNA clone is characterized
by the lack of a sequence encoding a signal peptide and by the lack of N-
linked glycosylation sites.

DNA sequences of the invention can be obtained by several methods. For
example, the DNA can be isolated using hybridization procedures which are
well known in the art. These include, but are not limited to : 1) hybridization
of probes to genomic or cDNA libraries to detect shared nucleotide sequences;
2) antibody screening of expression libraries to detect shared structural
features and 3) synthesis by the polymerase chain reaction (PCR).

Hybridization procedures are useful for the screening of recombinant clones
by using labeled mixed synthetic oligonucleotide probes where each probe is
potentially the complete complement of a specific DNA sequence in the

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hybridization sample which includes a heterogeneous mixture of denatured double-stranded DNA. For such screening, hybridization is preferably performed on either single-stranded DNA or denatured double-stranded DNA. Hybridization is particularly useful in the detection of cDNA clones derived from
5   sources where an extremely low amount of mRNA sequences relating to the polypeptide of interest are present. In other words, by using stringent hybridization conditions directed to avoid non-specific binding, it is possible, for example, to allow the autoradiographic visualization of a specific cDNA clone by the hybridization of the target DNA to that single probe in the mixture
10   which is its complete complement (Wallace, et al., Nucleic Acid Research, 9:879, 1981).

A NACP containing cDNA library can be screened by injecting the various mRNA derived from cDNAs into oocytes, allowing sufficient time for expression of the cDNA gene products to occur, and testing for the presence of the
15   desired cDNA expression product, for example, by using antibody specific for NACP or the X or Y peptide fragments thereof polypeptide or by using probes for the repeat motifs and a tissue expression pattern characteristic of NACP. Alternatively, a cDNA library can be screened indirectly for NACP polypeptides having at least one epitope using antibodies specific for the polypeptides, such
20   as X and Y peptides. Such antibodies can be either polyclonally or monoclonally derived and used to detect expression product indicative of the presence of NACP cDNA.

Screening procedures which rely on nucleic acid hybridization make it possible to isolate any gene sequence from any organism, provided the appropriate
25   probe is available. Oligonucleotide probes, which correspond to a part of the sequence encoding the protein in question, can be synthesized chemically.

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This requires that short, oligopeptide stretches of amino acid sequence must be known. The DNA sequence encoding the protein can be deduced from the genetic code, however, the degeneracy of the code must be taken into account. It is possible to perform a mixed addition reaction when the sequence is degenerate. This includes a heterogeneous mixture of denatured double-stranded DNA.

For such screening, hybridization is preferably performed on either single-stranded DNA or denatured double-stranded DNA. Hybridization is particularly useful in the detection of cDNA clones derived from sources where an extremely low amount of mRNA sequences relating to the polypeptide of interest are present. In other words, by using stringent hybridization conditions directed to avoid non-specific binding, it is possible, for example, to allow the autoradiographic visualization of a specific cDNA clone by the hybridization of the target DNA to that single probe in the mixture which is its complete complement (Wallace, et al., Nucl. Acid Res., 9:879, 1981).

The development of specific DNA sequences encoding NACP, or fragments thereof, can also be obtained by: 1) isolation of double-stranded DNA sequences from the genomic DNA; 2) chemical manufacture of a DNA sequence to provide the necessary codons for the polypeptide of interest; and 3) in vitro synthesis of a double-stranded DNA sequence by reverse transcription of mRNA isolated from a eukaryotic donor cell. In the latter case, a double-stranded DNA complement of mRNA is eventually formed which is generally referred to as cDNA. Of these three methods for developing specific DNA sequences for use in recombinant procedures, the isolation of genomic DNA isolates is the least common. This is especially true when it is desirable

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to obtain the microbial expression of mammalian polypeptides due to the presence of introns.

The synthesis of DNA sequences is frequently the method of choice when the entire sequence of amino acid residues of the desired polypeptide product is

5   known. When the entire sequence of amino acid residues of the desired polypeptide is not known, the direct synthesis of DNA sequences is not possible and the method of choice is the synthesis of cDNA sequences. Among the standard procedures for isolating cDNA sequences of interest is the formation of plasmid- or phage-carrying cDNA libraries which are derived from

10   reverse transcription of mRNA which is abundant in donor cells that have a high level of genetic expression. When used in combination with polymerase chain reaction technology, even rare expression products can be cloned. In those cases where significant portions of the amino acid sequence of the polypeptide are known, the production of labeled single or double-stranded

15   DNA or RNA probe sequences duplicating a sequence putatively present in the target cDNA may be employed in DNA/DNA hybridization procedures which are carried out on cloned copies of the cDNA which have been denatured into a single-stranded form (Jay, et al., Nucl. Acid Res., 11:2325, 1983).

A cDNA expression library, such as lambda gt11, can be screened indirectly

20   for NACP or NAC peptides having at least one epitope, using antibodies specific for NACP or the NAC peptide. Such antibodies can be either polyclonally or monoclonally derived and used to detect expression product indicative of the presence of NACP cDNA.

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DNA sequences encoding NACP or NAC can be expressed *in vitro* by DNA transfer into a suitable host cell. "Host cells" are cells in which a vector can be propagated and its DNA expressed. The term also includes any progeny of the subject host cell. It is understood that all progeny may not be identical to

5   the parental cell since there may be mutations that occur during replication. However, such progeny are included when the term "host cell" is used. Methods of stable transfer, in other words when the foreign DNA is continuously maintained in the host, are known in the art.

In the present invention, the NACP or NAC polynucleotide sequences may be

10   inserted into a recombinant expression vector. The term "recombinant expression vector" refers to a plasmid, virus or other vehicle known in the art that has been manipulated by insertion or incorporation of the NACP genetic sequences. Such expression vectors contain a promoter sequence which facilitates the efficient transcription of the inserted genetic sequence of the host.

15   The expression vector typically contains an origin of replication, a promoter, as well as specific genes which allow phenotypic selection of the transformed cells. Vectors suitable for use in the present invention include, but are not limited to the T7-based expression vector for expression in bacteria (Rosenberg, *et al., Gene, 56*:125, 1987), the pMSXND expression vector for

20   expression in mammalian cells (Lee and Nathans, *J. Biol. Chem., 263*:3521, 1988) and baculovirus-derived vectors for expression in insect cells. The DNA segment can be present in the vector operably linked to regulatory elements, for example, a promoter (e.g., T7, metallothionein I, or polyhedrin promoters and enhancer).

Polynucleotide sequences encoding NACP or NAC peptides can be expressed in either prokaryotes or eukaryotes. Hosts can include microbial, yeast, insect and mammalian organisms. Methods of expressing DNA sequences having eukaryotic or viral sequences in prokaryotes are well known in the art.

5   Biologically functional viral and plasmid DNA vectors capable of expression and replication in a host are known in the art. Such vectors are used to incorporate DNA sequences of the invention.

Transformation of a host cell with recombinant DNA may be carried out by conventional techniques as are well known to those skilled in the art. Where

10   the host is prokaryotic, such as *E. coli*, competent cells which are capable of DNA uptake can be prepared from cells harvested after exponential growth phase and subsequently treated by the $CaCl_2$ method by procedures well known in the art. Alternatively, $MgCl_2$ or RbCl can be used. Transformation can also be performed after forming a protoplast of the host cell or by

15   electroporation.

When the host is a eukaryote, such methods of transfection of DNA as calcium phosphate co-precipitates, conventional mechanical procedures such as microinjection, electroporation, insertion of a plasmid encased in liposomes, or virus vectors may be used. Eukaryotic cells can also be cotransformed with

20   DNA sequences encoding the NACP or NAC proteins of the invention, and a second foreign DNA molecule encoding a selectable phenotype, such as the herpes simplex thymidine kinase gene. Another method is to use a eukaryotic viral vector, such as simian virus 40 (SV40) or bovine papilloma virus, to transiently infect or transform eukaryotic cells and express the protein.

25   (*Eukaryotic Viral Vectors*, Cold Spring Harbor Laboratory, Gluzman ed., 1982).

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Isolation and purification of microbially expressed polypeptide, or fragments thereof, provided by the invention, may be carried out by conventional means including preparative chromatography and immunological separations involving monoclonal or polyclonal antibodies.

5   One skilled in the art will also be able to create a living mouse model for expressing the NACP gene in a living transgenic mouse. Methods of making a transgenic mouse expressing a foreign gene utilize several techniques for inserting the foreign gene into the germline of the animal at an early developmental stage, such as at the single-cell level. For instance, the

10   transgene can be inserted into a mouse oocyte, which is then implanted into mouse for birth of a transgenic animal. See U. S. Patent No. 4,873,191, "Genetic Transformaion of Zygotes," which is incorporated herein in its entirety. Similarly, pluripotent embroyo-derived stem (ES) cells, can be modified extra-corporeally by insertion of a cloned gene to transfer a modification to the germ

15   line of a living organism.

Homologous recombination has also been used for targeting genetic mutations to a predetermined genetic locus of an ES cell in order to produce a transgenic animal (Mansour, et al., Nature, 336:348, 1988; Capecchi, M., Trends Genet., 5:70, 1989). Homologous recombination between DNA sequences

20   residing in the chromosome and newly introduced cloned DNA sequences allows the transfer of any modification to the cloned gene into the genome of a living cell. Several site-specific recombination systems are known (Craig, Ann. Rev. Genet., 22:77, 1988) including the FLP system of yeast and the Cre system of bacteriophage P1. The FLP recombinase of the yeast

25   saccharomyces cerevisiae acts on copies of a recombination target called FRTs. The FLP system has been shown to effect site-specific recombination

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in the *Drosophilia* genome *in vivo* (Golic, *et al., Cell,* 59:499, 1989, Golic, K., *Science,* 252:958, 1991) and in eukaryotic cells *in vitro* (O'Gorman, *et al., Science,* 251:1351, 1991).

5   A novel approach to effecting specific homologous recombination events in eukaryotes is the prokaryotic Cre-loxP site-specific DNA recombination system of coliphage P1. The 38 kD Cre protein efficiently causes both inter- and intra-molecular recombination between specific 34 base pair repeats termed loxP (Sternberg, *et al., J. Mol. Biol.,* 150:467, 1981). Each loxP site contains two 13 base pair inverted repeats and an 8 base pair asymmetric core sequence.

10 No accessory proteins are required for exchange to occur. Direct repeats of loxP dictate an excision of intervening sequences while inverted repeats specify inversion. Cre has been shown to be functional in eukaryotic cells (Sauer, *et al., Nucleic Acids Res.,* 27:147, 1989) and in transgenic plants (Dale, *et al., Proc. Natl. Acad. Sci. USA,* 88:10558, 1991).

15 These and other types of "gene targeting" provide a means for controlling the site of integration (Smithies, *et al., Nature,* 317:230, 1985). For homologous recombination to occur between two DNA molecules, the molecules must possess a region of sequence identity with respect to one another, typically several hundred base pairs in length. This method requires that the gene of 20 interest must have been previously cloned, and the intron-exon boundaries determined, as is the case herein. Targeted insertion increases the probability that an inserted gene will function as desired. It also reduces the chance of random insertion activating a quiescent oncogene or inactivating a cancer supressor gene.

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U. S. Patent Nos. 5,175,383, 5,175,381 and 5,175,385, which are incorporated

herein by reference in their entirety, illustrate utilization of these methods of

targeted insertion to either correct or mutate a desired chromosomal locus,

thereby creating a transgenic animal. U. S. Patent No. 5,175,383 discloses the

5   method of making a transgenic mouse model for the human disease benign

prostatic hypertrophy. To create the Harvard mouse the int-2 oncogene, which

codes for a growth factor, was joined to a control gene to ensure that the

growth factor would be produced in prostate tissue. Systems for studying

regulation of genes in transgenic animal are also disclosed in Patent

10   Application WO 90/06367 entitled "Transgenic Mice for the Analysis of Hair

Growth" wherein insertion of a promoter of a gene for a hair specific protein,

one expressed only in tissues involved in hair growth, is used to regulate

expression of a reporter gene. Due to conservation among homologous genes

and their products, transgenes can be expressed in mice under the control of

15   a regulatory sequence from a human tissue specific gene. Recently, Patent

Application WO 93/14200, which is incorporated herein by reference in its

entirety, discloses creation of a trangenic mouse that expresses β-amyloid

precursor proteins.

D.   METHODS FOR USE OF NAC ANTISENSE POLYNUCLEOTIDES

20   The NAC polynucleotide in the form of an antisense polynucleotide is useful in

treating disease states associated with formation of amyloid i.e., amyloidosis

in the brain, (particularly in neuritic) plaques by preventing expression of the

protein that is originating. Essentially, any disorder which is etiologically linked

to expression of NACP could be considered susceptible to treatment with a

25   reagent of the invention which modulates NACP expression. The term

"modulate" envisions the suppression of expression of NACP when it is

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over-expressed, or augmentation of NACP expression when it is under-expressed or when the NACP expressed is a mutant form of the polypeptide.

5   When amyloidosis is associated with NACP overexpression, such suppressive reagents as antisense NACP polynucleotide sequence or NACP binding antibody can be introduced to a cell. Alternatively, when an amyloid disorder is associated with underexpression or expression of a mutant NACP polypeptide, a sense polynucleotide sequence (the DNA coding strand) or NACP polypeptide can be introduced into the cell. Methods for use of

10   antisense gene therapy are discussed in greater detail below.

E.   ANTI-NAC AND ANTI-NACP ANTIBODIES.

The invention includes polyclonal and monoclonal antibodies immunoreactive with NACP or NAC polypeptides or immunogenic fragments thereof.

Antibodies which are specific for NAC or NACP may be produced by

15   immunization of a non-human with antigenic NAC or NACP peptides of native or synthetic origin. Once antigenic peptides are prepared, antibodies to the immunizing peptide are produced by introducing peptide into a mammal (such as a rabbit, mouse or rat).

A multiple injection immunization protocol is preferred for use in immunizing

20   animals with the antigenic MTA peptides (see, e.g., Langone, et al., eds., "Production of Antisera with Small Doses of Immunogen: Multiple Intradermal Injections", Methods of Enzymology (Acad. Press, 1981). For example, a good antibody response can be obtained in rabbits by intradermal injection of 1 mg

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of the antigenic MTA peptide emulsified in Complete Freund's Adjuvant followed several weeks later by one or more boosts of the same antigen in Incomplete Freund's Adjuvant.

If desired, the immunizing peptide may be coupled to a carrier protein by
5 conjugation using techniques which are well-known in the art. Such commonly used carriers which are chemically coupled to the peptide include keyhole limpet hemocyanin (KLH), thyroglobulin, bovine serum albumin (BSA), and tetanus toxoid. The coupled peptide is then used to immunize the animal (e.g. a mouse or a rabbit).

10 Polyclonal antibodies produced by the animals can be further purified, for example, by binding to and elution from a matrix to which the peptide to which the antibodies were raised is bound. Those of skill in the art will know of various techniques common in the immunology arts for purification and/or concentration of polyclonal antibodies, as well as monoclonal antibodies (see,
15 for example, Coligan, et al., Unit 9, Current Protocols in Immunology, Wiley Interscience, 1991).

If desired, polyclonal antibodies can be further purified, for example, by binding to and elution from a matrix to which NAC polypeptide is bound. Those of skill in the art will know of various other techniques common in the immunology arts
20 for purification and/or concentration of polyclonal antibodies, as well as monoclonal antibodies.

Antibodies which consist essentially of pooled monoclonal antibodies with different epitopic specificities, as well as distinct monoclonal antibody preparations are provided. Monoclonal antibodies are made from antigen

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containing fragments of the protein by methods well known to those skilled in the art (Kohler, et al., Nature, 256:495, 1975). The term antibody or, immunoglobulin as used in this invention includes intact molecules as well as genetically engineered antibody constructs such as bifunctional antibodies,

5   CDR grafted antibodies, and the like, as well as fragments thereof, such as Fab and F(ab')$_2$, which are capable of binding an epitopic determinant on NACP or NAC.

A preferred method for the identification and isolation of an antibody binding domain that exhibits binding with NACP or NAC peptides is the bacteriophage

10   λ vector system. This vector system has been used to express a combinatorial library of Fab fragments from the mouse antibody repertoire in Escherichia coli (Huse, et al., Science, 246:1275-1281, 1989) and from the human antibody repertoire (Mullinax, et al., Proc. Natl. Acad. Sci., 87:8095-8099, 1990). As described therein, receptors (Fab molecules) exhibiting binding for a

15   preselected ligand were identified and isolated from these antibody expression libraries. This methodology can also be applied to hybridoma cell lines expressing monoclonal antibodies with binding for a preselected ligand. Hybridomas which secrete a desired monoclonal antibody can be produced in various ways using techniques well understood by those having ordinary skill

20   in the art and will not be repeated here. Details of these techniques are described in such references as Monoclonal Antibodies-Hybridomas: A New Dimension in Biological Analysis, Edited by Roger H. Kennett, et al., Plenum Press, 1980; and, U.S. Patent No. 4,172,124.

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F.   METHODS FOR DETECTING AMYLOID OR A CELL EXPRESSING
      NACP.

The invention provides a method for detecting a cell expressing NACP, or an

amyloid disorder associated with NAC, comprising contacting a cell suspected

5   of expressing NACP or having a NAC associated disorder with a reagent which

binds to the target component. The cell component can be nucleic acid, such

as DNA or RNA, or protein. When the component is nucleic acid, the reagent

is a nucleic acid probe or PCR primer. When the cell component is protein,

the reagent is an antibody probe. The probes are detectably labeled, for

10   example, with a radioisotope, a fluorescent compound, a bioluminescent

compound, a chemiluminescent compound, a metal chelator or an enzyme.

Those of ordinary skill in the art will know of other suitable labels for binding

to the antibody or probe, or will be able to ascertain such, using routine

experimentation.

15   For purposes of the invention, an antibody or nucleic acid probe specific for

NACP or fragments thereof may be used to detect the presence of NACP

polypeptide or NAC peptides (using antibody) or polynucleotide (using nucleic

acid probe) in biological fluids or tissues.   Any specimen containing a

detectable amount of antigen can be used.   A preferred sample in this

20   invention is tissue of brain origin, specifically midfrontal cortex tissue obtained

through biopsy. More preferably, the tissue is hippocampus tissue. Preferably

the subject is human.

Another technique which may also result in greater sensitivity consists of

coupling the antibodies to low molecular weight haptens. These haptens can

25   then be specifically detected by means of a second reaction. For example, it

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is common to use such haptens as biotin, which reacts with avidin, or dinitrophenyl, pyridoxal, and fluorescein, which can react with specific anti-hapten antibodies.

The method for detecting a cell expressing NACP or a amyloid disorder
5   associated with NAC, described above, can be utilized for prescreening for detection of amyloidosis prior to or after a subject's manifestation of typical clinical and neuropathological features of AD.   Additionally, the method for detecting NACP polypeptide in cells is useful for prescreening to detect risk of amyloid disorder by identifying cells expressing NACP at levels different than
10 normal cells. Using the method of the invention, high, low, and mutant NACP expression can be identified in a cell and the appropriate course of treatment can be employed (e.g., sense or antisense gene therapy).

The monoclonal antibodies of the invention are suited for use, for example, in immunoassays in which they can be utilized in liquid phase or bound to a solid
15 phase carrier. In addition, the monoclonal antibodies in these immunoassays can be detectably labeled in various ways.   Examples of types of immunoassays which can utilize monoclonal antibodies of the invention are competitive and non-competitive immunoassays in either a direct or indirect format. Examples of such immunoassays are the radioimmunoassay (RIA) and
20 the sandwich (immunometric) assay. Detection of the antigens using the monoclonal antibodies of the invention can be done utilizing immunoassays which are run in either the forward, reverse, or simultaneous modes, including immunohistochemical assays on physiological samples. Those of skill in the art will know, or can readily discern, other immunoassay formats without undue
25 experimentation.

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The monoclonal antibodies of the invention can be bound to many different carriers and used to detect the presence of NACP or NAC peptides such as X and Y.   Examples of well-known carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified
5   celluloses, polyacrylamides, agaroses and magnetite. The nature of the carrier can be either soluble or insoluble for purposes of the invention. Those skilled in the art will know of other suitable carriers for binding monoclonal antibodies, or will be able to ascertain such using routine experimentation.

As used in this invention, the term "epitope" includes any determinant capable
10   of specific interaction with the monoclonal antibodies of the invention. Epitopic determinants usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics.

15   The concentration of detectably labeled monoclonal antibody which is administered should be sufficient such that the binding to those cells having NAC or NACP is detectable compared to the background.   Further, it is desirable that the detectably labeled monoclonal antibody be rapidly cleared from the circulatory system in order to give the best target-to-background
20   signal ratio.

Because of the blood/brain barrier, it can be expected that antibodies will not be particularly the preferred reagent for use in *in vivo* applications. Rather, NAC/NACP polypeptides (particularly the former) that will cross the blood-brain barrier, and bind to the native protein are expected to be the best NAC/NACP
25   ligands. In particular, the preferred ligands of the invention will be those which

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are derived from positions 61-95 of the NACP amino acid sequence (i.e., in the NAC region), and from the C-terminal region of NACP (positions 131-140). In part, this preference is derived from the observation that antibodies to NAC (61-95) bind amyloid plaques, while antibodies to NACP (131-140) bind synapses, as well as the presumed ability of the peptides to cross the blood brain burner.

It has been shown that β-amyloid peptide fragments of about 28 amino acids in length or shorter will effectively cross the blood/brain barrier *in vivo* without toxic effect (see, e.g., Examples 15 and 16, as well as co-pending, commonly owned U.S. Patent Application No. 08/136,751; filed 10/14/93). Given the similarity in structure between the pleated β-amyloid molecule and NAC (see, Example 8, below), it can be expected that NAC peptides of about 28 amino acids or shorter in length would cross the blood-brain barrier. As shown in Example 8, NAC is a self-aggregating peptide (which apparently derives from the 61-95 amino acid region of NACP). Within the 61-95 stretch of amino acids (see, SEQ.ID.No.1), the following peptides have been determined to have self-aggregating ability (i.e., binding sites for NAC) using the method described in Example 8 (reading from the N to the C terminus):

TVEGAGSIAAATGFVKKD (NAC peptide 1)

and   KKKTVEGAGSIAAATGFV (NAC peptide 2).

Further, the somewhat shorter NACP peptides described below (which are derived from the 131-140 region of NACP; see, SEQ.ID.No.1), would also be expected to cross the blood/brain barrier (reading from the N to the C terminus):

EGYQDYEPEAKKD (NACP peptide 1)

and KKKEGYQDYEPEA (NACP peptide 2).

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Determination of whether a particular NAC or NACP peptide will specifically bind NAC or NACP can be readily made without undue experimentation by one of ordinary skill in the art. In regard to characterstics of peptides that may

5   cross the blood/brain barrier, those of skill in the art may wish to refer to Pardridge, WA, "*Peptide Drug Delivery to the Brain*", (Raven Press, 1991), chapters 3, 6 and 7 of which in particular are incorporated herein by this reference to illustrate the state of knowledge in the art concerning delivery of peptides to the brain. An example of a suitable animal model and testing

10   protocol for use in this regard are set forth in Examples 15-16.

As an illustration of techniques which may be employed to identify peptides that may cross the blood/brain barrier, without undue experimentation, peptides shorter than NAC (61-95) or NACP (131-140) can be screened for use in the

15   method of the invention by incubation with AD brain tissue homogenates or brain tissue from an animal model which has been implanted surgically with amyloid, or through immunological techniques such as those described above (e.g., testing the reactivity of anti-NACP antibodies that react with the native protein to the candidate ligand).

20   It is also possible to determine without undue experimentation if a NAC or NACP peptide (i.e., NAC or NACP candidate ligand) has the same specificity as the NAC ligand described above by ascertaining whether the former prevents the latter from binding to NAC. If the candidate ligand competes with a ligand which is known to bind NAC/NACP (as shown by a decrease in

25   binding by the latter), then the two peptides bind to the same, or a closely related site.

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Still another way to determine whether a particular candidate ligand has the specificity of a ligand which is known to bind NAC/NACP is to preincubate the candidate ligand with NAC or NACP and then add a known ligand to determine if it is inhibited in its ability to bind NAC or NACP. If the known NAC/NACP

5   ligand is inhibited, in all likelihood the candidate ligand has the same, or functionally equivalent, binding specificity as the known NAC/NACP ligand.

Screening of candidate ligands can also be determined by attaching a detectable label to them, incubating them with amyloid-containing brain tissue (*in vivo* or *in vitro*) and determining whether binding has occurred using *in vivo*

10   diagnostic imaging techniques as described in more detail below.

NAC and NACP ligands may be labelled as described below; however, for *in vivo* diagnostic imaging, the use of radiolabels or paramagnetic isotopes will be preferred. For example, for *in vivo* diagnostic imaging, the type of detection instrument available is a major factor in selecting a given radioisotope. The

15   radioisotope chosen must have a type of decay which is detectable for a given type of instrument. Still another important factor in selecting a radioisotope for *in vivo* diagnosis is that the half-life of the radioisotope be long enough so that it is still detectable at the time of maximum uptake by the target, but short enough so that deleterious radiation with respect to the host is minimized.

20   Ideally, a radioisotope used for *in vivo* imaging will lack a particle emission, but produce a large number of photons in the 140-250 keV range, which may be readily detected by conventional gamma cameras.

For *in vivo* diagnosis, radioisotopes may be bound to proteinaceous ligands either directly or indirectly by using an intermediate functional group.

25   Intermediate functional groups which often are used to bind radioisotopes

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which exist as metallic ions to immunoglobulins are the bifunctional chelating agents such as diethylenetriaminepentacetic acid (DTPA) and ethylenediaminetetraacetic acid (EDTA) and similar molecules. Typical examples of metallic ions which can be bound to the ligands of the invention are ^{111}In, ^{97}Ru, ^{67}Ga, ^{68}Ga, ^{72}As, ^{89}Zr, and ^{201}Tl. However, for its relatively low toxicity and ready imaging, [TM^{99}] (pertechenetate) will be the most preferred radiolabel for its relatively low toxicity in mammals. Radiolabelling with ^{99m}Tc may be performed according to the technique described in Kasnia, *et al. J. Nucl. Med.*, <u>32</u>:1445-1451, 1991.

However, for any *in vitro use*, ^{125}Iodide (^{125}I) would be preferred for ease of detection. ^{125}I may be attached to a NAC or NACP ligand for use in the invention by conventional techniques including oxidative radiodination using sodium ^{125}I and chloramine T (for tyrosine containing peptides) or the acylation followed by oxidative radiodination (for peptides not containing tyrosine). Iodination may also be performed using an iodination product from DuPont of Wilmington, DE (marketed under the trademark NEN) or the iodogen technique described in Salacinski, *et al.*, *Anal. Biochem.*, <u>117</u>:136-146, 1981. Iodogen for use in this method is commercially available from Pierce and Wariner, Chester, England.

The ligands of the invention can also be labeled with a paramagnetic isotope for purposes of *in vivo* diagnosis, as in magnetic resonance imaging (MRI) or electron spin resonance (ESR). In general, any conventional method for visualizing diagnostic imaging can be utilized. Usually gamma and positron emitting radioisotopes are used for camera imaging and paramagnetic isotopes for MRI. Elements which are particularly useful in such techniques include ^{157}Gd, ^{55}Mn, ^{162}Dy, ^{52}Cr, and ^{56}Fe.

Regardless of the detectable label used, the labelled ligands will preferably be purified by means well known in the art referred to above (for example, RP-HPLC) to an essentially quantitative specific activity (e.g., about 2000 Ci/mmol; 1Ci≈ 37GBq).

5   To practice the invention, a diagnostically effective amount of a detectably labelled ligand as described above will be administered to mammal which is suspected of having AD, has been diagnosed as having AD or, in the research context, has had amyloid plaque formation induced in its brain tissue. In the preferred embodiment, the mammal will be a human who is suspected of

10   having or has been diagnosed as having AD.

As a rule, the dosage of detectably labeled ligand for *in vivo* diagnosis will vary depending on such factors as age, sex, and extent of disease of the individual. The dosage of monoclonal antibody can vary from about 0.001 mg/m^2 to about 500 mg/m^2, preferably 0.1 mg/m^2 to about 200 mg/m^2, most preferably about

15   0.1 mg/m^2 to about 10 mg/m^2. Such dosages may vary, for example, depending on whether multiple injections are given, amyloid burden, and other factors known to those of skill in the art.

Those skilled in the art will be able to determine an appropriate dosage for the detectably labelled ligands based on the animal study data provided in the

20   examples below. In general, the "diagnostically effective amount" of detectably labelled NAC ligand for *in vivo* applicants will be that amount which is sufficient to detectibly bind any NAC present in the subject brain tissue.

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Although binding of the detectably labelled peptides of the invention is somewhat dose-dependent, it will be appreciated because the peptides are self-aggregating, increasing their dosage may intensify rather than expand the NAC binding pattern. More specifically, while binding of most or all of the NAC

5   plaques present in the subject brain tissue may occur at lower dosage levels, the intensity of the emissions indicative of that binding may be enhanced as the detectably labelled peptide density per plaque is increased at higher dosage levels.

Although any nonsurgical route of administration which introduces the

10   detectably labelled ligands of the invention into brain tissue may be used, intraarterial injection in preferred, with intracarotid injections being most pre-ferred. Where the method of the invention is being performed for diagnostic purposes, the background measurement will preferably be established by determining the extent of binding of a detectably labelled ligand in healthy

15   mammalian subjects. In this context, "healthy" is defined as a mammal with less than about 15 amyloid plaques/unit area of brain tissue (one unit area = 0.1 square millimeter) and/or a subject who exhibits no clinical signs of a neuropsychological disorder. In the same regard, measurements based on binding of the detectably labelled ligand indicative of the presence of ≥ about

20   15 plaques/unit area will be considered to be diagnostically significant for (i.e., indicative of) AD. These data can be used to assist in confirmation or refutation of a clinical diagnosis of AD.

To evaluate the prognosis of a subject who is suspected of having or has been diagnosed as having AD, the method of the invention can provide data of at

25   least three significant types. First, using NAC ligand, plaque density in excess of about 15 plaques/unit area can be correlated to the progress of the disease

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(i.e., greater plaque density would be expected to be present in later stages of the disease). Second, measurements of plaque development taken over time will be indicative of the rate at which the disease is progressing and/or of the effectiveness of a particular treatment or therapy. In applying the inventive

5   method to evaluate the progression of the disease, previous plaque density measurements taken from that subject would be used as background. Third, using NACP ligand, the probable progression of loss of cognitive function as well as the status of plaque formation through NACP cleavage, may be evaluated based on decreses in the synaptic population and/or increases in

10   presynatpic bouton concentrations of NACP.

Binding will preferably be measured within one hour of introducing the detectably labelled peptide into the subject's bloodstream. Binding will be measured *in vivo* using well-known *in vivo* diagnostic imaging techniques (in particular computer assisted sectional radiography (tomography)), preferably

15   during the first hour following administration of the detectably labelled peptide.

Of the presently known tomography techniques, positron emission tomography (PET) and single photon emission computed tomography (SPECT) are preferred for use in the method of the invention. Because the appropriate use of these techniques will be known or apparent to those skilled in the art, their

20   use will not be described in detail here.

For both prognosis and diagnosis, it may be desirable to evaluate the results of the *in vivo* binding assay of the invention in combination with evidence of the synaptic integrity of the subject brain tissue as well as clinical signs of disease. One suitable *in vitro* technique for evaluating and detecting synaptic loss in

25   sections of brain tissue using anti-synaptophysin antibodies is described in

Masliah, *et al., Am. J. Pathol.,* 137:1293-1297 (1990), the disclosure of which is hereby incorporated by reference to demonstrate techniques for evaluating synaptic loss known in the art; other techniques will be known to those of skill in the neurological arts.

5   For research purposes, the NAC/NACP ligands may be used for *in vitro* studies, for example, binding affinity of different peptides, to develop anti-amyloid antibodies, to study the pathology of amyloid deposition and to evaluate proposed therapies. The NAC/NACP peptides may be of particular use in developing *in vivo* means of differential diagnosis. For example,

10   because NAC principally appears in mature plaques, the stage of AD development is a given patient may be identified more accurately by detecting NAC *in vivo* than is now possible using conventional diagnostic techniques.

More generally, the NAC/NACP ligands of the invention can be used to monitor the course of amelioration of NAC associated amyloid disorder. Thus, by

15   measuring the increase or decrease in the number of cells expressing NACP or changes in the concentration of normal versus mutant NACP or NAC present in various body fluids and/or tissues, it would be possible to determine whether a particular therapeutic regiment aimed at ameliorating the disorder is effective.

20   G.   THERAPEUTIC METHODS FOR TREATING NAC ASSOCIATED AMYLOID DISORDER.

The present invention also provides a method for treating a subject with a NAC associated amyloid disorder. Because the NACP nucleotide sequence can be expressed in an altered manner as compared to expression in a normal cell,

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it is possible to design appropriate therapeutic or diagnostic techniques directed to this sequence. Thus, where an amyloid disorder is associated with the over-expression of NACP, nucleic acid sequences that interfere with NACP expression at the translational level can be used. This approach utilizes, for

5   example, antisense nucleic acid and ribozymes to block translation of a specific NACP mRNA, either by masking that mRNA with an antisense nucleic acid or by cleaving it with a ribozyme. In cases when a amyloid disorder or abnormal cell phenotype is associated with the under expression of NACP or expression of a mutant NACP polypeptide, nucleic acid sequences encoding NACP

10   (sense) could be administered to the subject with the disorder.

Further, as indicated in the Background of the Invention, studies regarding accumulation of β-amyloid in brain tissue have indicated that binding of synthetic β-amyloid peptide to native β-amyloid actually retards the

15   accumulation of the native protein. Based on these results, and given the physical and functional relationships between NAC and β-amyloid (see, Examples 12 and 13 below), it can be expected that administration of NAC peptides will provide a therapeutic benefit to a person suffering from a NAC associated amyloid disorder, such as AD. NAC peptides that will cross the

20   blood-brain barrier and bind to NAC (to "modulate" the accumulation thereof) are identified elsewhere above, as are means to identify any additional NAC peptides possessing this ability.

Antisense nucleic acids are DNA or RNA molecules that are complementary to at least a portion of a specific mRNA molecule (Weintraub, *Scientific American*,

25   262:40, 1990). In the cell, the antisense nucleic acids hybridize to the corresponding mRNA, forming a double-stranded molecule. The antisense nucleic acids interfere with the translation of the mRNA since the cell will not

translate a mRNA that is double-stranded. Antisense oligomers of about 15
nucleotides are preferred, since they are easily synthesized and are less likely
to cause problems than larger molecules when introduced into the target
NACP-producing cell. The use of antisense methods to inhibit the *in vitro*
5   translation of genes is well known in the art (Marcus-Sakura, *Anal. Biochem.*,
172:289, 1988).

Ribozymes are RNA molecules possessing the ability to specifically cleave
other single-stranded RNA in a manner analogous to DNA restriction
endonucleases. Through the modification of nucleotide sequences which
10   encode these RNAs, it is possible to engineer molecules that recognize specific
nucleotide sequences in an RNA molecule and cleave it (Cech, *J. Amer. Med.
Assn.*, 260:3030, 1988). A major advantage of this approach is that, because
they are sequence-specific, only mRNAs with particular sequences are
inactivated.

15   There are two basic types of ribozymes namely, *tetrahymena*-type (Hasselhoff,
Nature, 334:585, 1988) and "hammerhead"-type. *Tetrahymena*-type ribozymes
recognize sequences which are four bases in length, while "hammerhead"-type
ribozymes recognize base sequences 11-18 bases in length. The longer the
recognition sequence, the greater the likelihood that that sequence will occur
20   exclusively in the target mRNA species. Consequently, hammerhead-type
ribozymes are preferable to *tetrahymena*-type ribozymes for inactivating a
specific mRNA species and 18-based recognition sequences are preferable to
shorter recognition sequences.

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The present invention also provides gene therapy for the treatment of amyloid disorders which are mediated by NACP protein. Such therapy would achieve its therapeutic effect by introduction of the NACP antisense polynucleotide, into target cells (i.e., in brain tissue) of subjects having the amyloid disorder.

5   Delivery of antisense NACP polynucleotide can be achieved using a recombinant expression vector such as a chimeric virus or a colloidal dispersion system. Disorders associated with under-expression of NACP could similarly be treated using gene therapy with sense nucleotide sequences.

Various viral vectors which can be utilized for gene therapy as taught herein
10   include adenovirus, herpes virus, vaccinia, or, preferably, an RNA virus such as a retrovirus. Preferably, the retroviral vector is a derivative of a murine or avian retrovirus. Examples of retroviral vectors in which a single foreign gene can be inserted include, but are not limited to: Moloney murine leukemia virus (MoMuLV), Harvey murine sarcoma virus (HaMuSV), murine mammary tumor
15   virus (MuMTV), and Rous Sarcoma Virus (RSV). A number of additional retroviral vectors can incorporate multiple genes. All of these vectors can transfer or incorporate a gene for a selectable marker so that transduced cells can be identified and generated. By inserting a NACP sequence of interest into the viral vector, along with another gene which encodes the ligand for a
20   receptor on a specific target cell, for example, the vector is now target specific. Retroviral vectors can be made target specific by inserting, for example, a polynucleotide encoding an enzyme that determines the structure of a sugar, a glycolipid, or a protein. Preferred targeting is accomplished by using an antibody to target the retroviral vector. Those of skill in the art will know of, or
25   can readily ascertain without undue experimentation, specific polynucleotide sequences which can be inserted into the retroviral genome to allow target

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specific delivery of the retroviral vector containing the NACP antisense polynucleotide.

Since recombinant retroviruses are defective, they require assistance in order to produce infectious vector particles. This assistance can be provided, for example, by using helper cell lines that contain plasmids encoding all of the structural genes of the retrovirus under the control of regulatory sequences within the LTR. These plasmids are missing a nucleotide sequence that enables the packaging mechanism to recognize an RNA transcript for encapsidation. Helper cell lines which have deletions of the packaging signal include but are not limited to ψ2, PA317 and PA12, for example. These cell lines produce empty virions, since no genome is packaged. If a retroviral vector is introduced into such cells in which the packaging signal is intact, but the structural genes are replaced by other genes of interest, the vector can be packaged and vector virion produced.

Alternatively, NIH 3T3 or other tissue culture cells can be directly transfected with plasmids encoding the retroviral structural genes *gag, pol* and *env*, by conventional calcium phosphate transfection. These cells are then transfected with the vector plasmid containing the genes of interest. The resulting cells release the retroviral vector into the culture medium.

Another targeted delivery system for NACP antisense polynucleotides is a colloidal dispersion system. Colloidal dispersion systems include macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. The preferred colloidal system of this invention is a liposome. Liposomes are artificial membrane vesicles which are useful as delivery vehicles

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in vitro and *in vivo*. It has been shown that large unilamellar vesicles (LUV), which range in size from 0.2-4.0 um can encapsulate a substantial percentage of an aqueous buffer containing large macromolecules. RNA, DNA and intact virions can be encapsulated within the aqueous interior and be delivered to

5   brain cells in a biologically active form (Fraley, *et al., Trends Biochem. Sci.*, 6:77, 1981). For instance *in vivo* administration can be in a bolus or by gradual perfusion over time by means adapted for crossing the blood-brain barrier. For instance, the NAC or NACP polynucleotides or polypeptides can be injected by epidural administration or intralumbar puncture using standard

10   techniques well known to the medical profession, although an intrarterial route of administration would be preferred for patient comfort.

In order for a liposome to be an efficient gene transfer vehicle, the following characteristics should be present: (1) encapsulation of the genes of interest at high efficiency while not compromising their biological activity; (2) preferential

15   and substantial binding to a target cell in comparison to non-target cells; (3) delivery of the aqueous contents of the vesicle to the target cell cytoplasm at high efficiency; and (4) accurate and effective expression of genetic information (Mannino, *et al., Biotechniques*, 6:682, 1988).

The composition of the liposome is usually a combination of phospholipids,

20   particularly high-phase-transition-temperature phospholipids, usually in combination with steroids, especially cholesterol. Other phospholipids or other lipids may also be used. The physical characteristics of liposomes depend on pH, ionic strength, and the presence of divalent cations.

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Examples of lipids useful in liposome production include phosphatidyl compounds, such as phosphatidylglycerol, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, sphingolipids, cerebrosides, and gangliosides. Particularly useful are diacylphosphatidylglycerols, where the lipid

5   moiety contains from 14-18 carbon atoms, particularly from 16-18 carbon atoms, and is saturated. Illustrative phospholipids include egg phosphatidylcholine, dipalmitoylphosphatidylcholine and distearoylphosphatidylcholine.

The targeting of liposomes has been classified based on anatomical and mechanistic factors. Anatomical classification is based on the level of

10   selectivity, for example, organ-specific, cell-specific, and organelle-specific. Mechanistic targeting can be distinguished based upon whether it is passive or active. Passive targeting utilizes the natural tendency of liposomes to distribute to cells of the reticulo-endothelial system (RES) in organs which contain sinusoidal capillaries. Active targeting, on the other hand, involves

15   alteration of the liposome by coupling the liposome to a specific ligand such as a monoclonal antibody, sugar, glycolipid, or protein, or by changing the composition or size of the liposome in order to achieve targeting to organs and cell types other than the naturally occurring sites of localization.

The surface of the targeted delivery system may be modified in a variety of

20   ways. In the case of a liposomal targeted delivery system, lipid groups can be incorporated into the lipid bilayer of the liposome in order to maintain the targeting ligand in stable association with the liposomal bilayer. Various linking groups can be used for joining the lipid chains to the targeting ligand.

The compounds bound to the surface of the targeted delivery system will generally be ligands and receptors which will allow the targeted delivery system to find and "home in" on the desired cells. A ligand may be any compound of interest which will bind to another compound; e.g., NAC.

5   In general, surface membrane proteins which bind to specific effector molecules are referred to as receptors. In the present invention, antibodies are preferred receptors. Antibodies can be used to target liposomes to specific cell-surface ligands. For example, certain antigenic sites associated with NAC polypeptides in amyloid formations may be exploited for the purpose of

10   targeting NAC polypeptide or polynucleotide containing liposomes directly to the amyloid deposit. Since the NACP gene product may be indiscriminate with respect to cell type in its action, a targeted delivery system offers a significant improvement over randomly administered non-specific liposomes. Preferably, the target tissue is brain tissue and the target cell is a neuron/glia.

15   A number of procedures can be used to covalently attach either polyclonal or monoclonal antibodies to a liposome bilayer. Antibody-targeted liposomes can include monoclonal or polyclonal antibodies or fragments thereof such as Fab, or F(ab')$_2$, as long as they bind efficiently to an antigenic epitope on the target cells. Liposomes may also be targeted to cells expressing receptors for

20   hormones or other serum factors.

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H.   KITS AND ASSAYS FOR USE IN THE METHODS OF THE INVENTION.

The antibodies and substantially purified NAC peptide of the present invention are ideally suited for the preparation of a kit. Such a kit may comprise a carrier means being compartmentalized to receive in close confinement

5   therewith one or more container means such as vials, tubes and the like, each of the container means comprising the separate elements of the assay to be used.

The types of assays which can be incorporated in kit form are many, and include, for example, competitive and non-competitive assays. Typical

10   examples of assays which can utilize the antibodies of the invention are radioimmunoassays (RIA), enzyme immunoassays (EIA), enzyme-linked immunosorbent assays (ELISA), and immunometric, or sandwich immunoassays.

The term "immunometric assay" or "sandwich immunoassay", includes

15   simultaneous sandwich, forward sandwich and reverse sandwich immunoassays. These terms are well understood by those skilled in the art. Those of skill will also appreciate that antibodies according to the present invention will be useful in other variations and forms of assays which are presently known or which may be developed in the future. These are intended

20   to be included within the scope of the present invention.

In performing the assays it may be desirable to include certain "blockers" in the incubation medium (usually added with the labeled soluble antibody). The "blockers" are added to assure that non-specific proteins, proteases, or anti-heterophilic immunoglobulins to anti-NACP or anti-NAC immunoglobulins

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present in the experimental sample do not cross-link or destroy the antibodies on the solid phase support, or the radiolabeled indicator antibody, to yield false positive or false negative results. The selection of "blockers" therefore may add substantially to the specificity of the assays described in the present invention.

5   It has been found that a number of nonrelevant (i.e., nonspecific) antibodies of the same class or subclass (isotype) as those used in the assays (e.g., IgG1, IgG2a, IgM, etc.) can be used as "blockers". The concentration of the "blockers" (normally 1-100 μg/μl) is important, in order to maintain the proper sensitivity yet inhibit any unwanted interference by mutually occurring cross

10   reactive proteins in the specimen.

I.   PHARMACEUTICAL COMPOSITIONS OF THE INVENTION.

The invention also relates to a method for preparing a medicament or pharmaceutical composition comprising the polynucleotides, monoclonal antibodies or the NAC/NACP ligands of the invention ("pharmaceutically active

15   molecules"), the medicament being used for therapy of NAC associated amyloid disorders.

Pharmaceutically active molecules will preferably be administered in a pharmaceutically acceptable carrier, which may include sterile aqueous of non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous

20   solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. A pharmaceutically active molecule of the invention

may be conjugated by means well known in the art to polyethylene glycol (PEG) to reduce its immunogenicity.

Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous
5   vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, antioxidants, chelating agents, and inert gases and the like.

The following examples illustrate the manner in which the invention can be
10   practiced. It is understood, however, that the examples are for the purpose of illustration and the invention is not to be regarded as limited to any of the specific materials or conditions therein.

EXAMPLE 1
AMYLOID PREPARATION

15   Amyloid was purified from the frontal cortex of patients with typical clinical and neuropathological features of AD (obtained from the Alzheimer's Disease Research Center, San Diego, CA) using a modification of a protocol previously described by J. Kondo, et al. (Neuron, 1:827-834, 1988). Briefly, AD cortex was homogenized in 2% SDS, 1% 2-mercaptoethanol, 50 mM Tris-HCl, pH 7.6,
20   heated to 95°C for 10 minutes, and then centrifuged at 100,000 x g for 60 minutes. The pellets were suspended in 50 mM Tris-HCl, pH 7.6, and 1% SDS (SDS buffer) and centrifuged at 100,000 x g for 60 minutes. The pellets were resuspended in 0.5 M sucrose in SDS buffer and subjected to 1.0/2.0 M sucrose step-gradient centrifugation at 245,000 x g for 2 hours. The interfaces

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were collected and centrifuged at 150,000 x g for 60 minutes after 5 times dilution with SDS buffer.

The pellets recovered were sonicated in SDS buffer, dialyzed against 70% formic acid, cleaved with CNBr, and digested with *Achromobacter lyticus*

5   protease I in 5 M urea at 30°C for 5 hours. The cleaved peptides were separated by HPLC on a C4 column with a linear gradient (0-80%) of acetonitrile/isopropanol (3/7) in 0.1% trifluoroacetic acid. All of the HPLC peaks eluted from HPLC were sequenced using methods known in the art. In addition to the major $A\beta$ sequence (31.1 nmol) two heretofore unknown

10   peptides X (2.0 nmol) and Y (2.3 nmol) were recovered. All of the amino acid sequences found could be attributed to proteins known to be associated with amyloid tissue--$A\beta$ protein, τ, ubiquitin, ferritin, and collagen--except for the two heretofore unknown peptides, named herein as NAC peptide X (SEQ. I.D. NO. 4) and NAC peptide Y (SEQ. I.D. NO. 5). No other sequences of NACP other

15   than peptides X and Y were detected in the amyloid preparation.

Because Peptides X and Y were recovered in essentially the same concentration, the hypothesis was drawn that they were derived from a single larger precursor peptide NACP. Because the amyloid fraction contained sequences of τ and ubiquitin, known components of PHF, it was possible that

20   the X and Y peptides might have derived from contaminating PHF. To exclude this possibility, it was necessary to localize X and Y with immunological probes.

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EXAMPLE 2

ANTIBODY PRODUCTION AND IMMUNOHISTOCHEMISTRY

Proteins X and Y were synthesized using methods known in the art, tested for solubility, and found not to be soluble in any aqueous solution examined.

5   Therefore, the N-terminal sequences of proteins X and Y were synthesized and used to raise rabbit antisera using methods known in the art. For example, a C-terminal cystein was added to the 9 N-terminal amino acids of protein X to form a protein fragment X1 EQVTNVGGAC (SEQ. I.D. NO. 6), and a C-terminal cystein was added to the 7 N-terminal amino acids of protein Y to form a

10   protein fragment Y (TVEGAGSC) (SEQ. I.D. NO. 7). These protein fragments X1 and Y were conjugated to KLH using MBS as described in N. Green, et al., (Cell 28:477-487, 1982). Rabbits were boosted several times with the MBS-conjugated peptides, and then proteins X and Y were conjugated to KLH with glutaraldehyde, injected into rabbits, and antisera were obtained as described

15   in E. Masliah, et al. (J. Neurosci., 10:2113-2124, 1990).

Antisera obtained from the rabbits were used to perform immunohistochemical and immunoelectron microscope analysis of AD brain sections using methods known in the art (Masliah, supra, 1990; E. Masliah, et al. (J. Neurosci., 11:2759-2767, 1991). In particular, specificity of the staining was demonstrated by a

20   pre-absorption experiment consisting of incubation of antibody X1 with peptide X1 and incubation of antibody Y with peptide Y. In the control, the antisera were incubated with peptide of an entirely different sequence (EGYQDYEPEAC) (SEQ. I.D. NO. 8).

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In order to define which structures were immunostained (neurofibrillary tangles, neuritic plaques, vascular amyloid, and neuropil threads) and to identify plaque subtypes (diffuse, primitive, and mature), some of the immunostained sections were further stained with a 1% aqueous solution of thioflavin S and were

5   viewed with ultraviolet illumination and fluorescein filters using procedures described in Masliah, *supra* (1991). The results were further confirmed by immunoelectron microscope analysis as described in Masliah, *supra*, (1991). Briefly, vibratome sections were blocked with normal goat serum (5%) and incubated overnight at 4°C with antibodies anti-X1 and anti-Y. The sections

10   were washed in PBS, incubated with biotinylated goat anti-rabbit IgG followed by avidin D-HRP (Vector ABC Elite, Vector Labs, Inc., Burlingame, CA) and reacted with diaminobenzidine (DAB, 0.2 mg/ml) in 50 mM Tris buffer (pH 7.4) with 0.001% H_2O_2. Control sections were incubated with preimmune serum. The immunostained sections were postfixed for 20 minutes in 1% OsO_4,

15   dehydrated, and embedded to present a flat surface in epoxy/Araldite. Ultrathin sections were cut with a Reichert OM-U3 ultramicrotome and viewed with a 100 CX JEOL electron microscope.

Results of the immunohistochemical and immunoelectron microscope analysis of AD brain sections are shown in FIGURE 1. Panel A shows hippocampal

20   sections stained with antibodies anti-X1 and anti-Y. In Panel B occasional staining of dystrophic neutites (arrows) was detected with anti-Y antibody. Immunostaining of amyloid in diffuse, primitive, and mature plaques was detected in Panels A and B. as well as in cerebral vessel walls (not shown) as revealed by double staining with thioflavin S. When these sera were

25   preabsorbed with fragments X1 and Y, staining was eliminated. As shown in Panel C, neither preimmune sera nor antisera containing anti-X1 and/or anti-Y antibodies stained amyloid in AD brain tissue when antisera had been

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preabsorbed with the corresponding peptide fragments X1 and Y. Panel D shows an electron micrograph of staining of amyloid fibrils (arrows) with anti-X1 antibody. Amyloid fibrils were also stained with the anti-Y antibody (not shown).

5   The anti-Y antibody stained not only amyloid in plaques, but also nuclei of small, possibly glial cells, cytoplasm of some small cells, and neuropil threads. The staining of these structures other than amyloid was not observed with the anti-X1 antibody. There are two potential explanations for the difference in staining properties between the anti-X1 and -Y peptide antibodies. This

10   difference in staining may represent a genuine difference in the distribution of two products of a single precursor protein or, alternatively, it may be a result of a less specific staining by the anti-Y peptide antibody. It appears likely that the latter explanation is correct because anti-Y antibody stains dozens of bands on Western blots of brain extract (data not shown).

15   Recent work has shown that 50% of intracellular NFTs and 100% of extracellular MFTs contain Aβ. (G. Perry, *et al., Am. J. Pathol.*, 140:283-290, 1992). However, the thioflavin-positive NFTs did not stain positively with anti-X1 or -Y antibodies.

       Immunoelectron microscopic analysis of QsO$_4$ intensified diaminobenzidine

20   staining by anti-X1 antibody showed specific localization on amyloid fibrils (Fig. 1D). Anti-Y antibody also stained amyloid fibrils (not shown). These results indicate that both X and Y peptides are tightly associated with the amyloid fibrils. The data are compatible with the hypothesis that NAC is actually a novel amyloid component and not a component of contaminants such as

25   paired helical filaments.

EXAMPLE 3

MOLECULAR CLONING OF NACP CDNA

To discover whether the NAC peptides are produced from a precursor protein as is the case with Aβ, the decision was made to isolate the cDNA. First a

5   piece of cDNA for encoding the NAC peptide was deduced and amplified by PCR as described in R. K. Saiki, et al. (Science, 230:1350-1354, 1985). Sense (X1) (SEQ. I.D. NO. 9) and antisense (X2) (SEQ. I.D. NO. 10) oligonucleotides were designed as primers for the N- and C-terminal halves of the X peptide amino-acid sequence, respectively.

10   An additional set of sense Z (SEQ. I.D. NO. 11) and antisense αZ (SEQ. I.D. NO. 12) oligonucleotide primers for DNA flanking the EcoRI cloning site of λgt11 were made. PCR was performed using these oligonucleotides as primers with combinations of X1 (or X2) and Z (or αZ) as primers and a cDNA expression library of human brain tissue in λgt11 as template. Briefly, the PCR

15   reaction was performed at 94°C for 1 minute, at 51°C for 1.5 minute, and at 72°C for 2 minutes. After 35 cycles of amplification, a PCR DNA product of about 280 bp obtained from the combination of primers X2 and αZ was purified, digested with EcoRI, subcloned into the EcoRI-HincII site of pBluescript SK+ (Stratagene, San Diego, CA), and sequenced using methods

20   well known in the art.

An amino acid sequence deduced from the DNA sequence thus obtained was found to contain a ten amino acid sequence (EQATNAGGVA) constituting the N-terminal region of X peptide, thus confirming the identity of this PCR product as a fragment of NACP cDNA. With the origin of the PCR product confirmed,

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the DNA fragment was used as a probe for screening a cDNA expression library of human brain tissue (ATCC, #37432) in λgt11 phage.

From 5 x 10^5 recombinants, 22 positive clones were obtained. The isolated cDNA were subcloned into pBluescript SK+ and sequenced on both strands

5   by Sanger dideoxy chain termination method (Sanger et al. 1977) using Sequenase (U. S. Biochemical Co., Cleveland, OH) with the help of synthetic primers. Clone HBS6-1 contained an apparently full-length cDNA with a nested sequence corresponding to NAC sequence.

Bacterial Expression of NACP Protein.

10   NACP protein was expressed in *E. coli* using pSENACP expression vector. Plasmid pHBS6-1 was digested with *Af/*II, treated with Klenow polymerase to generate a blunt end, and then digested with *Noc*I to release the coding region. This 1.2-kb *Nco*I-*Af/*II NACP cDNA fragment containing the entire coding sequence and 3' nontranslated region was ligated into a bacterial

15   expression vector, pSE380 (Invitrogen), previously linearized by digestion with *Nco*I and *Sma*I. Resultant pSENACP expresses NACP protein under the control of trp/lac fusion promoter which is inducible with IPTG. pSENACP was amplified in *E. coli* HB101.

Northern Blot Analysis.

20   Procedures for RNA preparation, electrophoresis, and hybridization are those routinely used. Briefly, total RNA was isolated from different human tissues. RMA (10 μg) was electrophoresed on a 1% formaldehyde-agarose gel and blotted to a nitrocellulose membrane. Hybridization was carried out in 50% formamide, 5 x SSPE, 5 x Denhardt's, 0.5% SDS, 100μg/ml denatured salmon

25   sperm DNA, and 10% dextran sulfate at 42°C, and exposed for 3 days at

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○

-80°C. A higher stringency wash of 0.1 x SSC, 0.1% SDS at 65°C was also employed.

Sequence analysis of the HBS6-1 clone revealed a 420 bp open-reading frame DNA sequence encoding 140 amino-acid residues with a calculated relative

5   molecular mass (M$_r$) of 14,459 (SEQ. I.D. NO. 1). As shown in FIGURE 2A, the sequence surrounding the predicted initiator methionine codon (GCCATGG) agrees with the Kozak consensus sequence as described by K. Kozak (*Nucleic Acid Res.*, 15:8125-8148, 1987). Also as shown in FIGURE 2A, the nearest in-frame stop codon was found 18 bp upstream of this ATG. The nearest in-

10   frame stop codon (TAA) upstream to the putative initiation methionine codon is marked by an asterisk. The termination codon is marked by two asterisks. Sequences for X and Y peptides are boxed. Polyadenylation signals are underlined.

The deduced amino acid sequence shows that the X and Y peptides are

15   located immediately next to each other in the middle of the precursor protein NACP. Neither an apparent signal peptide sequence nor canonical N-linked glycosylation sites were found.

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EXAMPLE 4

1.   STUDIES OF THE AMINO ACID SEQUENCE TO DETERMINE INDICATIONS PREDICTIVE OF SECONDARY STRUCTURE.

Using the methods of P. Y. Chou, et al., Annu. Rev. Biochem., 47:251-276 (1978); G. D. Rose, Nature, 272:586-590 (1978); J. Garnier, et al., J. Mol. Biol., 120:97-120 (1978), studies were conducted to determine sequence features predictive of secondary structure. These studies indicate that the NAC peptide sequence has a strong tendency to form a β-sheet configuration, as does Aβ.

2.   HYDROPATHY TESTING OF THE PRECURSOR PROTEIN NACP.

Using the method of Kyte and Doolittle (J. Kyte and R.F. Doolittle, J. Mol. Biol., 157:105-132, 1982) an analysis of NACP was performed using a window size 9 and Prosis software from Pharmacia (Piscataway, NJ). The main hydrophobic domain of the deduced amino acid sequence (amino acids 62 through 90) was located within the sequence of the NAC proteins X and Y (amino acids 61 through 95). When the NAC protein was synthesized, aggregates and precipitates formed easily in aqueous solutions. These results indicate that the precursor protein NACP is considerably more soluble in aqueous solutions (cytoplasm and other bodily fluids) than are the X and Y peptides associated with amyloid formations.

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EXAMPLE 5

WESTERN BLOT ANALYSIS OF NACP PROTEIN.

Tissue homogenates were prepared from cytosolic fractions of frontal cortex from patients with typical and neuropathological features of AD obtained from
5   the Alzheimer's Disease Research Center (San Diego, CA). Procedures for human brain preparation, electrophoresis, and immunoblotting detection have been reported by E. Masliah, et al. (J. Neurosci, 10:2113-2124, 1990). Briefly, proteins were electrophoresed on a 16% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. Immunoblotting detection was
10   performed using anti-X1 antibody (1:1500 dilution) and ^{125}I-protein A. To absorb anti-X1 antibody, 200 μg/ml X1 fragment (SEQ. I.D. NO. 6) or the control peptide (EGYQDYEPEAC) (SEQ. I.D. NO. 8) were used.

Western blot analysis with anti-X1 antibody detected NACP as a M_r 19K protein mostly in the cytosolic fractions as shown in FIGURE 3, lane 4. Lanes 1 and
15   6 of FIGURE 3 show E. coli transfected with pSE380 vector as control; lanes 2 and 5 show E. coli transfected with pSENACP expressing NACP; and lanes 3 and 4 show normal human brain. A shorter exposure time was employed for lanes 1 to 3 compared with lanes 4 to 6 because of high background. However, the M_r 19K band was not observed in lanes 2 and 3 even after a 4
20   times longer exposure.

The anti-Y antibody stained dozens of bands in addition to the M_r 19K band (data not shown). Protein staining of the M_r 19K band was abolished when either the anti-X1 or the anti-Y antibody was preabsorbed with its corresponding peptide fragment as shown in FIGURE 3, lane 3. However, the

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anti-X1 positive M_r 40 band was not blocked by preabsorption and was not detected with anti-Y antibody, indicating that this band is not specific to NACP.

To confirm the identity of the protein derived from brain homogenates, a NACP protein derived from the cDNA isolated in Example 3 above was produced *in vitro*. pHBS6-1 was digested with AflII, treated with Klenow polymerase to generate a blunt end, and then digested with NcoI to release the coding region. This 1.2kb NcoI-AflII NACP cDNA fragment of Sequence I. D. No. 1 containing the entire coding sequence and 3'-nontranslated region was ligated into a bacterial expression vector, pSE380 (Invitrogen, San Diego, CA), previously linearized by digestion with NcoI and SmaI. The resultant vector pSENACP expresses NACP protein under the control of trp/lac fusion promoter which is inducible with IPTG. pSENACP was amplified in E. coli HB101 using techniques as described in Sambrook, *supra* and others. A vector pSE380 without NACP cDNA was also transfected in E coli as a control. In Western Blot analysis as shown in FIGURE 3, lane 3, the bacterially expressed protein of M_r 19K comigrated with the homogenate-derived protein, confirming the identity of the homogenate-derived protein.

EXAMPLE 6

SEQUENCE ANALYSIS OF NACP PROTEIN

The amino acid sequence of NACP was examined for distinctive structural features. As shown in FIGURE 4A, the NACP protein is characterized by repetitive motifs. The KTKEGV motif is repeated seven times, but the amino acid positions 2 to 6 are sometimes substituted. In addition, as shown in FIGURE 4B, amino acids 48-56 and 70-78 of the NACP protein are homologous.

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In addition to these repetitive motifs within the NACP protein, a computer homology search (FASTA program, in the UCSD BAX/VMS DNA protein sequence analysis system) of the DNA sequence data base (EMBL/GenBank Libraries) has discovered homology between NACP and EST01420, a protein

5   recently identified by random sequencing of human brain cDNA (M. D. Adams, *et al.*, *Nature*, 355:632-634, 1992). Comparison of the DNA encoding these proteins expressed in the human brain, as shown in FIGURE 4C, showed 74% identity in 124 nucleotides resulting in 80% identity in a 41 amino acid residue from the initiation methionine. Therefore, the two cDNAs are substantially

10   homologous in the N-terminal region, but the EST01420 sequence has the termination codon at base pair position 206, and, therefore, could encode only 51 amino acids. The high degree of homology in the cDNA encoding these peptides expressed in the brain suggests that NACP may be a member of a heretofore unknown gene family.

15   **EXAMPLE 7**

                    NORTHERN BLOT ANALYSIS OF NACP MRNA

Procedures for RNA preparation, electrophoresis, and hybridization have been described previously (T. Saitoh, *et al.*, *Cell*, 58:615-622, 1989). Briefly, total RNA was isolated from different human tissues as described in Chirgwin, J.M.,

20   *et al.* (*Biochemistry*, 18:5294-5299, 1979). RNA (10 μg) was electrophoresed on a 1% formaldehyde-agarose gel and blotted to a nitrocellulose membrane. Hybridization was carried out in 50% formamide, 5 x SSPE, 5 x Denhardt's, 0.5% SDS, 100 μg/ml denatured salmon sperm DNA, and 10% dextran sulfate

at 42°C for 15 hr with ^{32}P-labeled 1.1-kb NACP cDNA which includes the X and Y sequence with a 3'-noncoding sequence. The membrane was washed with 0.1 x SSPE, 0.1% SDS at 42°C, and exposed for 3 days at -80°C. A higher stringency wash of 0.1 x SSC, 0.1% SDS at 65°C was also employed. A

5   Northern blot was obtained from Clontech (Palo Alto, CA, #7760-1, Lot 32409). Hybridization and washing conditions for this blot followed the suggestions of the manufacturer.

As shown in FIGURE 5, Panel A was probed with NACP cDNA and, as a control, panel B was probed with glyceraldehyde-3-phosphate dehydrogenase

10   (GAPD) cDNA (ATCC, #57090). Briefly, the membrane was washed with 0.1 x SSPE, 0.1% SDS at 42°C, and exposed for 3 days at -80°C. The same pattern of signals was obtained after a higher stringency wash with 0.1 x SSC, 0.1% SDS at 65°C.

Two principal transcripts of 3.6 and 1.5 kb were found most enriched in brain,

15   with lower concentrations in all tissues examined except in liver as shown in FIGUREs 5A and 5B. Comparable patterns of mRNA were observed in both normal and AD neocortex and cerebellum as shown in FIGURE 5D. Since HBS6-1 is 1560 nucleotides long it probably corresponds to the shorter transcript. A minor 1 kb band visible in FIGURE 5 D is believed to represent

20   a transcript with a shorter 3' non-coding region resulting from the use of polyadenylation signals at 1023 bp or 1079 bp. It was discovered that the ratio of the different-sized transcripts varies depending on the age and origins of tissue employed as shown in FIGURE 5. The presence of the 3.6 kb transcript for NACP may be explained by an alternative splicing mechanism that is under

25   developmental and/or tissue specific regulation.

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EXAMPLE 8

NAC PEPTIDE AGGREGATION AND WESTERN BLOT ANALYSIS

A solution of synthetic NAC peptide (SEQ.ID.No.3) at a concentration of 600 μM was made in 50 mM boric buffer at pH 9.2. The solution was centrifuged at 100,000 x g

5   for 1 hr and the supernatant was collected, neutralized by 1.5 N hydrochloride in 10 x PBS, and diluted in 1 x PBS (pH 7.4).

The aggregation of synthetic NAC peptide was followed by measurement of turbidity according to techniques known in the art (see, e.g., Jarrett, et al., Biochemistry, 31:12345-12352 (1992); and, Jarrett, et al., Cell, 73:1055-1058 (1993)). Turbidity was

10 measured at 400 nm daily for 7 days under the following two conditions: 1) various concentrations of NAC peptide: 10, 30, 100, 300 μM at 37°C, and 2) 300 μM of NAC peptide at various temperatures: 4°, 22°, and 37°C.   The peptide solutions/suspensions were mixed gently before each absorbance measurement.

The size of the aggregated NAC peptide was estimated by Western blot using

15 anti-NAC-X1.   Six aliquots of peptide solution were prepared in boric buffer, lyophilized, and dissolved in water, neutralized, and diluted with PBS (phosphate buffered saline) to a final concentration of 300 μM. They were kept at 37°C for 0, 1, 2, 3, 5, or 7 days, dissolved in Laemmli sample buffer (Laemmli, U. K., Nature, 227:680-685 (1970)), electrophoresed on 16% Tricine-SDS-polyacrylamide gel, and

20 transferred to PVDF membrane for Western blot analysis.

NAC peptide was solubilized in 50 mM boric buffer (pH 9.2) although it was also soluble in formic acid and 6 M guanidinium thiocyanate. NAC peptide aggregated in distilled water and PBS.   Turbidity at 400 nm of NAC peptide solution in PBS increased with time. As shown in FIGURE 6 A, this increase was dependent on both

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the concentration of NAC peptide and the temperature (FIGURE 6 B). At Day 1, 300 μM NAC peptide was required for the appreciable aggregation, whereas at Day 2 100 μM NAC peptide started to aggregate. The aggregation of NAC peptide at less than 30 μM was never remarkable. The aggregation of NAC peptide at 22 °C was

5   comparable to that at 37 °C, although at 4 °C the aggregation was markedly delayed.

In Western blot, the monomer NAC peptide migrated to the apparent molecular mass of 3500 Da (FIGURE 7). The signal intensity of the 3500 Da band was significantly decreased on Day 5 and 7. Aggregated NAC peptide was found at the top of the gel from Day 1. This signal increased to a maximum at Day 2-3. No intermediate-size

10   bands detected by anti-NAC-X1 were observed. Thus, anti-NAC-1 recognized NAC peptide but not its precursor, NACP, in both dot and Western blot analysis. These findings indicate that this antiserum is sensitive to both the sequence and the conformation, allowing the detection of only NAC peptide but NACP that surely contains the NAC peptide sequence. However, anti-NAC-X1 is not simply the

15   conformational antibody, because it did not detect Ab blotted under the comparable conditions. Anti-NAC-X1 stained amyloid cores in AD brain. These data indicate that NAC fragments, which are shorter than NACP and have the similar structure as NAC peptide, accumulate in amyloid, and that they are immunologically identical to NAC peptide but distinct from NACP.

20   **EXAMPLE 9**

                          CONGO RED STAINING AND BIREFRINGENCE

Four hundred microliters of NAC peptide solution/suspension (300 μM) at pH 7.4 was stored over 2 weeks at 37°C to promote its aggregation. The solution/suspension was centrifuged at 16,000 x g for 30 min. The precipitate of peptide fibrils was

25   collected, mixed in 1 ml of PBS solution containing 1 mM Congo red for 1 min,

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centrifuged at 16,000 x g for 5 min, and recollected. It was rinsed with 1 ml of distilled water for 1 min, and centrifuged at 16,000 x g for 5 minutes. The peptide precipitate was placed on a glass microscope slide and allowed to dry. Birefringence was determined with an Olympus fluorescence microscope (model BHF) equipped
5   with a polarizing filter apparatus (model BH-POL).

The NAC peptide aggregate was stained by Congo red. It exhibited green-gold birefringence when viewed with bright-field (FIGURE 8 A) and cross-polarization (FIGURE 8 B) microscopy. The stained peptides appear in the FIGURES as bright patches.

10   EXAMPLE 10

ELECTRONMICROSCOPIC OBSERVATION

Electron microscopy of aggregated NAC peptide revealed clusters of fibrils deposited from the peptide suspension (FIGURE 9). The diameter of fibrils was generally about an Å. The repeated structures were found in the fibrils. The structure of NAC peptide
15   fibrils was different from that of Ab in AD brain.

Based on the above-referenced data, it was determined that the NAC peptide has the following characteristics of amyloid: 1) green birefringence after Congo red staining when viewed with a polarizing microscope; 2) a typical structure under the electron
20   microscope (fine, rigid, nonbranching fibrils); 3) insolubility in aqueous solution. Further, NAC was determined to be self-aggregating.

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However, it is important to note that the amyloid found in the brain tissue of patients with AD when viewed with electron microscopy is different from that formed by NAC aggregation. Thus, it is likely that NAC can not explain the global process of amyloid formation in AD. Rather, it is speculated that NAC is involved in only the initial process of amyloid formation and the major process of amyloidogenesis is due to the accumulation of Ab aggregation. Thus, NAC may serve as a seed to form amyloid as a minor component, on which Ab aggregate as a major component of amyloid. Further investigation will investigate the possibility that NAC may contribute to the process of Ab aggregation and amyloid forming and maturing.

EXAMPLE 11

DISTRIBUTION OF NACP IN RAT BRAIN SECTIONS

Using the streptavidin-biotin-peroxidase (SAB) staining method, rat brain sections were stained by anti-NACP(131-140; SEQ.ID.No.6) and anti-NACP(1-9; SEQ.ID.No.8) antisera. Briefly, Sprague-Dawley rats weighing 250 - 300 g were deeply anesthetized by sodium pentobarbital (Nembutal, Abbott Laboratories, North Chicago, IL), perfused transcardially with 4% paraformaldehyde in PBS for 20 min, and the brain was removed and immediately placed in a postfixative, 4% paraformaldehyde solution, for 4 days at 4°C. The brain was immersed in a 30% sucrose solution in PBS for 4 days at 4°C, frozen in -40°C hexane, and cut into 20-μm sections using a cryostat at -20°C.

Immunohistochemistry was performed according to the modified SAB method. Sections were rinsed for 3 x 5 min in PBS, incubated for 10 min in PBS including 0.1% Triton X-100, and incubated for 20 min with 3% H2O2 to inhibit endogenous peroxidase. They were then incubated for 10 min with 10% normal goat serum (NGS) (Nichirei, Tokyo, Japan) in PBS and incubated for 18 hr at 4°C with

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anti-NACP(131-140) or anti-NACP(1-9) antiserum in PBS containing 1% BSA. Sections were rinsed for 3 x 5 min in PBS and incubated with biotinylated goat anti-rabbit-IgG solution (Nichirei, Tokyo, Japan) for 10 min. They were then rinsed for 3 x 5 min in PBS, incubated with streptavidin-peroxidase solution (Nichirei, Tokyo, Japan) for 5

5   min, and rinsed for 3 x 5 min in PBS. NACP-positive structures were visualized by incubating the tissue in 0.05% diaminobenzidine with 0.01% H_2O_2 in 0.61 M Tris/HCl buffer (pH 7.4) for 5 - 15 min. Specificity of the immunohistochemical reaction was confirmed by the absence of staining in adjacent tissue sections incubated with preabsorbed antiserum.

10   Double-immunostaining was performed with antisera against NACP and synaptophysin as described previously (Masliah, et al., Exp.Neurol., 113:131-142, 1991). After treatment with 0.1% Triton X-100 and 3% H_2O_2, sections were incubated for 1 hr with 5% normal horse serum (NHS) (Vector Labs, Inc., Burlingame, CA), 10% NGS (Vector Labs, Inc.), and 2% BSA in PBS. They were then incubated

15   for 18 hr at 4°C with the mixture of mouse monoclonal antibody against synaptophysin (Sy38, Boehringer Mannheim, Indianapolis, IN) (Wiedenmann and Franke, Cell, 41:1017-1028, 1985) and rabbit polyclonal antisera, anti-NACP(131-140) or anti-NACP(1-9), in PBS containing 3% NHS, NGS, and BSA. Sections were rinsed for 3 x 5 min in PBS, incubated for 1 hour with biotinylated goat anti-rabbit-IgG in PBS

20   including 1% BSA, and rinsed for 3 x 5 min in PBS. They were then incubated for 80 minutes with Texas-red-labeled avidin (Vector Labs, Inc.) and fluorescein isothiocyanate (FITC)-labeled horse anti-mouse-IgG antibody (Vector Labs, Inc.) in PBS including 1% BSA and rinsed for 3 x 5 min in PBS. The double-labeled sections were covered with glass coverslips with antifading medium (Vector Labs, Inc.).

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These sections were observed with the Bio-Rad MRC-600 laser confocal scanning microscope mounted on a Nikon Optiphot microscope. This system permits the simultaneous analysis of double-labeled samples in the same optical plane. The digitized video images of serial 1-μm optical sections were stored on an optical disk

5   for subsequent processing and analysis.

For electron microscopic study, 40-μm vibratome sections were blocked with NGS (5%) and incubated overnight at 4°C with anti-NACP(131-140). The sections were washed in PBS, incubated with biotinylated goat anti-rabbit IgG followed by avidin D-HRP (Vector ABC elite, Vector Labs, Inc.), and reacted with diaminobenzidine (DAB;

10   0.2 mg/ml) in 50 mM Tris buffer (pH 7.4) with 0.001% H2O2. The immunostained sections were postfixed for 20 min in 1% OsO4, dehydrated, and flat embedded in epoxy/Araldite. Ultrathin sections were cut with a Reichert OM-U3 ultramicrotome and viewed with a 100 CX JEOL electron microscope.

15   The intensity of staining with anti-NACP(131-140) was relatively strong in the gray matter of the cerebral cortex (layers II, III, and V), anterior olfactory nucleus, caudate putamen, nucleus accumbens, hippocampus, ventral tegmental area, substantia nigra, pontine nuclei, and cerebellar cortex as shown macroscopically in FIGURE 10 A. The staining pattern of sections using anti-NACP(1-9) antisera was identical to that of

20   anti-NACP(131-140). This staining was abolished by preincubation of the antisera with the corresponding peptide.

The results of microscopic observation of the sections stained using the SAB method are shown in FIGURES 10 B-D. In cerebellum (FIGURE 10 B), the molecular layer and granule cell layer were stained, but the medullary layer and the Purkinje cell perikarya

25   were not stained. In both the molecular and granule cell layers, the perikarya of cells, including stellate, basket, Golgi, granule, and glial cells were not stained. An

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intermediate region where neurites were extended was stained with a "punctate" pattern. In hippocampus (FIGURE 10 C) and cerebral cortex (FIGURE 10 D), cell perikarya were not stained and neurites were stained showing "punctate" staining pattern similar to the molecular layer of the cerebellum. No ependymal or mantle
5   cells were stained in any region.

Laser scanning confocal microscopic analysis of sections double immunolabeled with antibodies against NACP and synaptophysin showed that both markers colocalized in the great majority of the presynaptic terminals (FIGURES 11 and 12). In the neocortex, hippocampus, basal ganglia, olfactory region, and thalamus, between 70
10   and 100% of the axosomatic, axoaxonic, and axodentritic terminals contained both markers (FIGURE 12). In contrast, in the deep cerebellar nuclei and brain stem nuclei, although synaptophysin and NACP colocalization in axodendritic terminals was relatively common, only a small proportion of the axosomatic terminals contained NACP (FIGURES 11 and 12). In both cortical and subcortical regions a small
15   proportion of terminals was synaptophysin positive and NACP negative (FIGURE 12). Occasionally, punctate structures displayed NACP immunoreactivity but not synaptophysin reactivity. Serial section analysis showed that these neuritic structures actually corresponded to the terminal segment of axons. Abundant NACP immunoreactivity was observed associated with specialized synaptic complexes
20   including the glomeruli of the olfactory bulb (FIGURE 11 D-F) and the glomeruli of the cerebellar granular layer (FIGURE 11 G-I). At the ultrastructural level (shown by immunoelectron microscopy), the membrane of synaptic vesicles in presynaptic terminals were stained by anti-NACP(131-140) (FIGURE 13).

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EXAMPLE 12

QUANTIFICATION OF NACP, APP AND SYNAPTOPHYSIN

IN RAT BRAIN SECTIONS

Brains of young adult Sprague-Dawley rats weighing 250 - 300 g were separated into

5   10 portions as follows: olfactory bulb, frontal cortex, striatum, hippocampus, hypothalamus, thalamus, midbrain, cerebellum, pons & medulla oblongata, and pituitary gland. For APP and synaptophysin quantification, 40 μg protein of either cytosolic or particulate fraction was loaded on a 10% SDS-polyacrylamide gel and blotted to nitrocellulose membrane. The mouse monoclonal antibody against the

10   N-terminal of APP (22C11) (Boehringer Mannheim) (Weidemann, *et al., Cell*, 57:115-126, 1989) or SY38 (Boehringer Mannheim) in PBS, including 1% BSA and 0.1% Tween20, was used as the primary antibody, followed by rabbit anti-mouse IgG polyclonal antibody (Accurate Chemical and Scientific Corp., Westbury, NY) at the dilution of 1:2000 in PBS including 3% BSA for 1 hr, then incubated with 0.5 μCi/ml

15   iodinated protein A and apposed to Kodak X-Omat RP film at -80°C.

Films were then developed with a Konica film developer and scanned with an LKB densitometer for quantification of the NACP bands and APP bands. X-ray film was exposed to membrane to give bands in the OD range between 0.8 and 2.5 where the sensitivity of the film is relatively linear.

20

Immunoreactive NACP protein bands were detected as a molecular mass of 19,000 Da in the cytosolic fraction of brain homogenate.   Since APP protein bands were observed at molecular masses of 75,000 - 105,000 Da in the cytosolic fraction and at molecular masses of 100,000 - 115,000 Da in the particulate fraction by 22C11, a

25   monoclonal antibody against APP (Weidemann, *et al.*, 1989, *supra*), the sum of signal intensity in both fractions was used as the amount of APP. Synaptophysin protein

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bands were observed with a molecular mass of 38,000 Da in the particulate fraction of brain sample by a mouse monoclonal antibody, SY38.

As shown in FIGURES 14 through 16, the concentration of NACP was high in the telencephalon (end-brain areas), including the olfactory bulb, frontal cortex, striatum,

5   and hippocampus, intermediate in the hypothalamus and thalamus, and low in the midbrain, cerebellum, pons & medulla oblongata, and pituitary gland. APP and synaptophysin were more evenly distributed in most portions of brain. However, the amount in the pituitary gland was small.

EXAMPLE 13

10   ## ALTERATION OF NACP IN AD BRAIN TISSUE

As described in Example 1, AD brain tissue was obtained from the frontal cortex of patients with typical clinical and neuropathological features of AD (supplied by the Alzheimer's Disease Research Center, San Diego, CA). Using the double immunolabeling and laser confocal microscopy techniques described in the preceding

15   Examples, the quantity of NACP in presynaptic terminals, as well as diffuse and mature amyloid plaques, was compared in tissue from AD brain and "normal" brain tissue.

Interestingly, the number of NACP containing presynptic terminals is significantly decreased in AD brain tissue as compared to normal brain tissue--by 30-40%

20   (FIGURES 15 through 16). Further, although the overall synaptic populations are decreased in AD brain tissue, there is a significant <u>increase</u> in the quantity of NACP present in each presynaptic bouton of AD brain tissue, indicating a compensatory mechanism for the loss of synapses (FIGURES 15 through 16).

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In addition, anti-NAC antibodies (as described elsewhere above) bound about 35% of the diffuse plaques and about 55% of the mature plaques present in the AD brain tissue tested (FIGURE 17). Double immunolabeling of tissue with anti-β-amyloid antibodies and anti-NAC antibodies showed that NAC is also more abundant than β-

5 amyloid in AD brain tissue (FIGURE 18). Further, control tissue samples obtained from elderly persons without AD but whose brain tissue contained relatively small groups of diffuse plaques did not react with anti-NAC antibodies, while tissue samples obtained from persons suffering from either early or advanced cases of AD that contained a relatively large number of diffuse plaques as well as mature plaques

10 reacted strongly with anti-NAC antibodies in about 30-50% of the plaques (FIGURES 16 and 18). These studies indicate that there is a connection between metabolism of presynaptic proteins (e.g., NACP) and plaque formation, and that NAC accumulation (in conjunction with β-amyloid accumulation) leads to the evolution of diffuse plaques into mature ones.

15   **EXAMPLE 14**

                    NACP HOMOLOGIES

As discussed in the Detailed Description of the Invention, a computer search of the DNA sequence data base (EMBL/ GenBank Libraries) revealed that some proteins had homologous sequence to NACP. Rat synuclein (SYN1) showed the highest

20 homology; 7 amino acids were substituted in 140 amino acid sequence in NACP. More specifically, the NACP amino acid sequence showed 95% identity with that of rat synuclein 1, a synaptic/nuclear protein previously identified in rat brain, indicating that NACP is the human homologue of rat synuclein 1. Rat SYN2 also showed high homology to NACP, howevere, approximate 50 amino acid sequence of C-terminal

25 was different. Rat SYN3 and human EST01420 identified by random sequencing of human brain cDNAs (Adams, et al., Nature, 355: 632-634, 1992), had homologous

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sequence to N-terminal portion of NACP, but neither of them included NAC portion. _Torpede_ synuclein also showed homologous sequence with 16 amino acids insertion between the residue 28 and 29 of NACP. Homo sapiens putatively transcribed partial sequence (HSPTPS), found by MRC Human Genome Mapping Project, had

5   homologous sequence corresponding to NAC portion of NACP. These data suggest that the metabolic alterations of presynaptic proteins are associated with the amyloid and plaque formation in Alzheimer's disease.

EXAMPLE 15

ANIMAL MODEL FOR _IN VIVO_ ADMINSTRATION

10   ## OF β-AMYLOID PEPTIDES USEFUL IN TESTING

THE ABILITY OF NAC/NACP PEPTIDES TO CROSS

THE BLOOD/BRAIN BARRIER IN MAMMALS

β-amyloid peptides of differing lengths as described below were administered to rats to determine whether the peptides would cross the blood/brain barrier in mammals.

15   The experimental animal models consisted of 6 groups as follows:

1) Rats injected unilaterally in the neocortex with 5 μl of full-length unlabeled β-amyloid (1-40, 0.01 mM, Bachem), followed 1 day later by intracarotid injection of ^{125}I substance P (100-500 pmol, NEN) or ^{125}I β-A peptide (1-28, 5 nmol, unlabeled peptide from Sigma Chemical Co. and iodination using DuPont's NEN iodination product); 2)

20   rats injected unilaterally in the neocortex with 5 μl of unlabeled β-amyloid (1-40, 0.01 mM) followed 1 day later by intracarotid injection of ^{125}I alone; 3) rats injected unilaterally in the neocortex with 5 μl of unlabeled β-amyloid (1-40, 0.01 mM), followed 1 day later by intracarotoid injection of ^{125}I substance P (100-500 pmol) or ^{125}I β-A peptide (1-28, 5 nmol); 4) rats injected unilaterally in the neocortex with 5 μl of sterile

25   saline, followed 1 day later by intracarotid injection of ^{125}I substance P (100-500 pmol)

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or ^{125}I β-A peptide (1-28, 5 nmol); **5)** rats injected unilaterally in the neocortex with 5 μl of sterile saline, followed 1 day later by intracarotid injection of sterile saline; **6)** rats injected unilaterally in the neocortex with 5 μl of unlabelled β-amyloid (1-40), followed 1 day later with labelled and unlabelled substance P and β-A peptide (1-28) injected

5   in the carotid artery.

Control data was generated from cryostat sections taken from the cortex of normal and AD cases which were incubated with radiolabelled and unlabelled β-amyloid and substance P. Use of intracerebral injections of amyloid into the rat brain as an animal model mimics the deposits of amyloid in the plaques of the patients with AD.

10   Although this animal model is not a model of AD *per se*, it is a model of amyloid deposits in the brain, which is one of the most important diagnostic hallmarks of AD. This animal model will, therefore, be useful in testing whether NAC/NACP peptides administered as described with respect to β-amyloid peptides will cross the blood/brain barrier.

15   **EXAMPLE 16**

β-A PEPTIDE (1-28) PASSAGE ACROSS THE BLOOD/BRAIN

BARRIER TO BIND UNLABELLED β-AMYLOID

Five hundred μl of ^{125}I β-amyloid (1-28) (5μCi/ml) were administered into the carotid arteries of rats. Five minutes after the injection about 100 CPM were measured in

20   brain samples. Maximum counts in the brain were recovered 50 minutes after injection because the number of CPM recovered from the brain reaches a plateau at this point in time.

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Rats that received direct injection of ^{125}Iβ-amyloid (1-28) into the brain displayed the highest CPM's recovered from the brain. Rats that received ^{125}I β-amyloid (1-28) into the carotid artery and that previously received an injection of unlabelled β-amyloid (1-40) into the neocortex displayed a 100 fold higher count in the brain compared with

5   rats that received an injection of saline alone in the brain followed by intracarotid administration of ^{125}I β-amyloid (1-28). Control experiments where ^{125}I β-amyloid (1-28) was substituted by vehicle alone showed only background CPM's, thus indicating that ^{125}I β-amyloid (1-28) injected into the circulation crossed the blood/brain barrier and bound the unlabelled amyloid injected in the brain.

10   As discussed in the Detailed Description of the Invention, these results indicate that NAC/NACP peptides of 28 amino acids in length or shorter will also cross the blood/brain barrier.

The CPM measurements reported were obtained by spectrophotometric analysis of fresh samples of brain tissue (taken from living, anaesthetized rats). For comparison,

15   cryostat sections were also taken from rats in each model group for *in vitro* analysis using the AMBIS 4000 radioimaging acquisition and analysis system (i.e., computed tomography system) (Ambis, San Diego, CA).

The foregoing description of the invention is exemplary for purposes of illustration and explanation. It should be understood that various modifications can be made without

20   departing from the spirit and scope of the invention. Accordingly, the following claims are intended to be interpreted to embrace all such modifications.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i) APPLICANT: THE REGENTS OF THE UNIVERSITY OF CALIFORNIA

    (ii) TITLE OF INVENTION: NOVEL COMPONENT OF AMYLOID IN
              ALZHEIMER'S DISEASE AND METHODS FOR USE OF SAME

    (iii) NUMBER OF SEQUENCES: 12

    (iv) CORRESPONDENCE ADDRESS:
        (A) ADDRESSEE: Spensley Horn Jubas & Lubitz
        (B) STREET: 1880 Century Park East - Suite 500
        (C) CITY: Los Angeles
        (D) STATE: California
        (E) COUNTRY: USA
        (F) ZIP: 90067

    (v) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Floppy disk
        (B) COMPUTER: IBM PC compatible
        (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

    (vi) CURRENT APPLICATION DATA:
        (A) APPLICATION NUMBER: PCT
        (B) FILING DATE: 29-AUG-1994
        (C) CLASSIFICATION:

    (viii) ATTORNEY/AGENT INFORMATION:
        (A) NAME: Howells, Stacy L.
        (B) REGISTRATION NUMBER: 34,842
        (C) REFERENCE/DOCKET NUMBER: FD-3520

    (ix) TELECOMMUNICATION INFORMATION:
        (A) TELEPHONE: (619) 455-5100
        (B) TELEFAX: (619) 455-5110

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(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 1560 base pairs
        (B) TYPE: nucleic acid
5   (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (vii) IMMEDIATE SOURCE:
        (B) CLONE: cDNA for NACP

10   (ix) FEATURE:
        (A) NAME/KEY: misc_RNA
        (B) LOCATION: 1..1560

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GCTCTCGGAG TGGCCATTCG ACGACAGTGT GGTGTAAAGG AATTCATTAG CCATGGATGT   60

15 ATTCATGAAA GGACTTTCAA AGGCCAAGGA GGGAGTTGTG GCTGCTGCTG AGAAAACCAA   120

ACAGGGTGTG GCAGAAGCAG CAGGAAAGAC AAAAGAGGGT GTTCTCTATG TAGGCTCCAA   180

AACCAAGGAG GGAGTGGTGC ATGGTGTGGC AACAGTGGCT GAGAAGACCA AAGAGCAAGT   240

GACAAATGTT GGAGGAGCAG TGGTGACGGG TGTGACAGCA GTAGCCCAGA AGACAGTGGA   300

GGGAGCAGGG AGCATTGCAG CAGCCACTGG CTTTGTCAAA AAGGACCAGT TGGGCAAGAA   360

20 TGAAGAAGGA GCCCCACAGG AAGGAATTCT GGAAGATATG CCTGTGGATC CTGACAATGA   420

GGCTTATGAA ATGCCTTCTG AGGAAGGGTA TCAAGACTAC GAACCTGAAG CCTAAGAAAT   480

ATCTTTGCTC CCAGTTTCTT GAGATCTGCT GACAGATGTT CCATCCTGTA CAAGTGCTCA   540

GTTCCAATGT GCCCAGTCAT GACATTTCTC AAAGTTTTTA CAGTGTATCT CGAAGTCTTC   600

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o

CATCAGCAGT GATTGAAGTA TCTGTACCTG CCCCCACTCA GCATTTCGGT GCTTCCCTTT   660

CACTGAAGTG AATACATGGT AGCAGGGTCT TTGTGTGCTG TGGATTTTGT GGCTTCAATC   720

TACGATGTTA AAACAAATTA AAAACACCTA AGTGACTACC ACTTATTTCT AAATCCTCAC   780

TATTTTTTTG TTGCTGTTGT TCAGAAGTTG TTAGTGATTT GCTATCATAT ATTATAAGAT   840

5   TTTTAGGTGT CTTTTAATGA TACTGTCTAA GAATAATGAC GTATTGTGAA ATTTGTTAAT   900

ATATATAATA CTTAAAAATA TGTGAGCATG AAACTATGCA CCTATAAATA CTAAATATGA   960

AATTTTACCA TTTTGCGATG TGTTTTATTC ACTTGTGTTT GTATATAAAT GGTGAGAATT   1020

AAAATAAAAC GTTATCTCAT TGCAAAAATA TTTTATTTTT ATCCCATCTC ACTTTAATAA   1080

TAAAAATCAT GCTTATAAGC AACATGAATT AAGAACTGAC ACAAAGGACA AAAATATAAA   1140

10   GTTATTAATA GCCATTTGAA GAAGGAGGAA TTTTAGAAGA GGTAGAGAAA ATGGAACATT   1200

AAGCCTACAC TCGGAATTCC CTGAAGCAAC ACTGCCAGAA GTGTGTTTTG GTATGCACTG   1260

GTTCCTTAAG TGGCTGTGAT TAATTATTGA AAGTGGGGTG TTGAAGACCC CAACTACTAT   1320

TGTAGAGTGG TCTATTTCTC CCTTCAATCC TGTCAATGTT TGCTTTATGT ATTTTGGGGA   1380

ACTGTTGTTT GATGTGTATG TGTTTATAAT TGTTATACAT TTTTAATTGA GCCTTTTATT   1440

15   AACATATATT GTTATTTTTG TCTCGAAATA ATTTTTTAGT TAAAATCTAT TTTGTCTGAT   1500

ATTGGTGTGA ATGCTGTACC TTTCTGACAA TAAATAATAT TCGACCATGA AAAAAAAAA   1560

(2) INFORMATION FOR SEQ ID NO:2:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 140 amino acids
20   (B) TYPE: amino acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: protein

(vii) IMMEDIATE SOURCE:
        (B) CLONE: NACP

(ix) FEATURE:
        (A) NAME/KEY: Protein
        (B) LOCATION: 1..140

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Asp Val Phe Met Lys Gly Leu Ser Lys Ala Lys Glu Gly Val Val
1               5                   10                  15

Ala Ala Ala Glu Lys Thr Lys Gln Gly Val Ala Glu Ala Ala Gly Lys
            20                  25                  30

Thr Lys Glu Gly Val Leu Tyr Val Gly Ser Lys Thr Lys Glu Gly Val
        35                  40                  45

Val His Gly Val Ala Thr Val Ala Glu Lys Thr Lys Glu Gln Val Thr
        50                  55                  60

Asn Val Gly Gly Ala Val Val Thr Gly Val Thr Ala Val Ala Gln Lys
65                  70                  75                  80

Thr Val Glu Gly Ala Gly Ser Ile Ala Ala Ala Thr Gly Phe Val Lys
                85                  90                  95

Lys Asp Gln Leu Gly Lys Asn Glu Glu Gly Ala Pro Gln Glu Gly Ile
                100                 105                 110

Leu Glu Asp Met Pro Val Asp Pro Asp Asn Glu Ala Tyr Glu Met Pro
                115                 120                 125

Ser Glu Glu Gly Tyr Gln Asp Tyr Glu Pro Glu Ala
                130                 135                 140
```

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(2) INFORMATION FOR SEQ ID NO:3:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 35 amino acids
        (B) TYPE: amino acid
5   (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (vii) IMMEDIATE SOURCE:
        (B) CLONE: NAC

10   (ix) FEATURE:
        (A) NAME/KEY: Peptide
        (B) LOCATION: 1..35

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Glu Gln Val Thr Asn Val Gly Gly Ala Val Val Thr Gly Val Thr Ala
1   5   10   15

Val Ala Gln Lys Thr Val Glu Gly Ala Gly Ser Ile Ala Ala Ala Thr
        20   25   30

Gly Phe Val
      35

20   (2) INFORMATION FOR SEQ ID NO:4:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 20 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
25   (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

```
     (vii) IMMEDIATE SOURCE:
            (B) CLONE: Peptide X

     (ix) FEATURE:
            (A) NAME/KEY: Peptide
 5          (B) LOCATION: 1..20


     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

     Glu Gln Val Thr Asn Val Gly Gly Ala Val Val Thr Gly Val Thr Ala
     1               5                   10                  15

     Val Ala Gln Lys
10                  20

     (2) INFORMATION FOR SEQ ID NO:5:

         (i) SEQUENCE CHARACTERISTICS:
               (A) LENGTH: 15 amino acids
               (B) TYPE: amino acid
15             (C) STRANDEDNESS: single
               (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: peptide


       (vii) IMMEDIATE SOURCE:
               (B) CLONE: Peptide Y

20     (ix) FEATURE:
               (A) NAME/KEY: Peptide
               (B) LOCATION: 1..15

       (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

       Thr Val Glu Gly Ala Gly Ser Ile Ala Ala Ala Thr Gly Phe Val
25     1               5                   10                  15
```

(2) INFORMATION FOR SEQ ID NO:6:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 9 amino acids
        (B) TYPE: amino acid
5   (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

   (vii) IMMEDIATE SOURCE:
        (B) CLONE: Fragment X1

10   (ix) FEATURE:
        (A) NAME/KEY: Peptide
        (B) LOCATION: 1..9

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

    Glu Gln Val Thr Asn Gly Gly Ala Cys
15   1   5

(2) INFORMATION FOR SEQ ID NO:7:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 8 amino acids
        (B) TYPE: amino acid
20   (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

   (vii) IMMEDIATE SOURCE:
        (B) CLONE: Fragment Y

25   (ix) FEATURE:
        (A) NAME/KEY: Peptide
        (B) LOCATION: 1..8

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

    Thr Val Glu Gly Ala Gly Ser Cys
    1   5

(2) INFORMATION FOR SEQ ID NO:8:

5   (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 11 amino acids
          (B) TYPE: amino acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

10   (ii) MOLECULE TYPE: peptide

    (vii) IMMEDIATE SOURCE:
          (B) CLONE: Control Peptide

    (ix) FEATURE:
          (A) NAME/KEY: Peptide
15   (B) LOCATION: 1..11

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

    Glu Gly Tyr Gln Asp Tyr Glu Pro Glu Ala Cys
    1   5   10

(2) INFORMATION FOR SEQ ID NO:9:

20   (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 29 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

25   (ii) MOLECULE TYPE: DNA (genomic)

    (vii) IMMEDIATE SOURCE:
          (B) CLONE: Sense Primer (X1)

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        (ix) FEATURE:
             (A) NAME/KEY: modified_base
             (B) LOCATION: 1..29
             (D) OTHER INFORMATION: /note= "where N is inosine"

 5   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GARCARGTNA CNAAYGTNGG NGGNGCNGT   29

     (2) INFORMATION FOR SEQ ID NO:10:

        (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 29 base pairs
10   (B) TYPE: nucleic acid
             (C) STRANDEDNESS: single
             (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA (genomic)

        (vii) IMMEDIATE SOURCE:
15   (B) CLONE: antisense primer (X2)

        (ix) FEATURE:
             (A) NAME/KEY: misc_RNA
             (B) LOCATION: 1..93

        (ix) FEATURE:
20   (A) NAME/KEY: modified_base
             (B) LOCATION: 1..29
             (D) OTHER INFORMATION: /note= "where N is inosine"

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TTYTGNGCNA CNGCNGTNAC NCCNGTNAC   29

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(2) INFORMATION FOR SEQ ID NO:11:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 23 base pairs
        (B) TYPE: nucleic acid
5   (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (vii) IMMEDIATE SOURCE:
        (B) CLONE: Sense Primer (z)

10 (ix) FEATURE:
        (A) NAME/KEY: misc_RNA
        (B) LOCATION: 1..23

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

ACGACTCCTG GAGCCCGTCA GTA   23

15 (2) INFORMATION FOR SEQ ID NO:12:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 23 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
20   (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (vii) IMMEDIATE SOURCE:
        (B) CLONE: Antisense Primer (alpha z)

    (ix) FEATURE:
25   (A) NAME/KEY: misc_RNA
        (B) LOCATION: 1..23

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GTAATGGTAG CGACCGGCGC TCA   23

SUMMARY OF THE SEQUENCES

Sequence I.D. No. 1 oligonucleotide is a sequence for cDNA encoding NACP polynucleotide.

Sequence I.D. No. 2 is an amino acid sequence for NACP polypeptide.

5   Sequence I.D. No. 3 is an amino acid sequence for NAC polypeptide.

Sequence I.D. No. 4 is an amino acid sequence for X peptide.

Sequence I.D. No. 5 is an amino acid sequence for Y peptide

Sequence I.D. No. 6 is an amino acid sequence for fragment X1

Sequence I.D. No. 7 is an amino acid sequence for fragment Y

10   Sequence I.D. No. 8 is an amino acid sequence for a control peptide.

Sequence I.D. No. 9 oligonucleotide is a sense primer (X1) for the N-terminal half of X peptide.

Sequence I.D. No. 10 oligonucleotide is an antisense primer (X2) for the C-terminal half of X peptide.

15   Sequence I.D. No. 11 oligonucleotide is a sense primer (Z) for a region flanking the EcoRI cloning site of lambda gt11.

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Sequence I.D. No. 12 oligonucleotide is an antisense primer (αZ) for a region
flanking the EcoRI cloning site of lambda gt11.

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CLAIMS

1.   An isolated polypeptide comprising all or a portion of NACP.

2.   An isolated polynucleotide which encodes the polypeptide of claim 1.

3.   The polynucleotide of claim 2, wherein the polynucleotide is DNA.

4.   The polynucleotide of claim 2, wherein the polynucleotide is RNA.

5.   A host cell containing the polynucleotide of claim 2.

6.   A recombinant expression vector containing the polynucleotide of claim 2.

7.   The vector of claim 6, wherein the polynucleotide is an antisense sequence.

8.   The vector of claim 6, which is a virus.

9.   The vector of claim 8, wherein the virus is an RNA virus.

10.   The vector of claim 9, wherein the RNA virus is a retrovirus.

11.   The vector of claim 6, wherein the vector is a colloidal dispersion system.

12. The vector of claim 11, wherein the colloidal dispersion system is a liposome.

13. The vector of claim 12, wherein the liposome is essentially target specific.

14. The vector of claim 13, wherein the liposome is anatomically targeted.

15. The vector of claim 13, wherein the liposome is mechanistically targeted.

16. The vector of claim 15, wherein the mechanistic targeting is passive.

17. The vector of claim 15, wherein the mechanistic targeting is active.

18. The vector of claim 17, wherein the liposome is actively targeted by coupling with a moiety selected from the group consisting of a sugar, a glycolipid and a protein.

19. The vector of claim 18, wherein the protein moiety is an antibody.

20. The vector of claim 6, wherein the vector is a plasmid.

21. Antibodies which are immunoreactive with the polypeptide of claim 1, or fragments thereof.

22. The antibodies of claim 21, wherein the antibodies are polyclonal.

23. The antibodies of claim 21, wherein the antibodies are monoclonal.

24. A method for detecting a cell expressing NACP comprising contacting a cell component with a reagent which binds to the component.

25. The method of claim 24, wherein the component is nucleic acid.

26. The method of claim 24, wherein the component is protein.

27. The method of claim 25, wherein the nucleic acid is DNA.

28. The method of claim 25, wherein the nucleic acid is RNA.

29. The method of claim 24, wherein the reagent is a probe.

30. The method of claim 29, wherein the probe is nucleic acid.

31. The method of claim 29, wherein the probe is an antibody.

32. The method of claim 31, wherein the antibody is polyclonal.

33. The method of claim 31, wherein the antibody is monoclonal.

34. The method of claim 26, wherein the protein is NACP and the reagent is an NACP peptide.

35. The method of claim 34, wherein the NACP peptide is about 28 amino acids in length or shorter.

36. The method of claim 26, wherein the protein is NACP and the reagent is a NAC peptide.

37. The method of claim 36, wherein the NAC peptide is about 28 amino acids in length or shorter.

38. The method of claim 24, wherein the reagent is detectably labeled.

39. The method of claim 38, wherein the label is selected from the group consisting of a radioisotope, a paramagnetic isotope, a bioluminescent compound, a chemiluminescent compound, a fluorescent compound, a metal chelate, or an enzyme.

40. A method for detecting amyloid formation in brain comprising contacting a brain tissue sample with a reagent which binds to NAC.

41. The method of claim 40, wherein the reagent is a probe.

42. The method of claim 41, wherein the probe is an antibody.

43. The method of claim 42, wherein the antibody is polyclonal.

44. The method of claim 42, wherein the antibody is monoclonal.

45. The method of claim 40, wherein the reagant is a NAC peptide.

46. The method of claim 45, wherein the NAC peptide is about 28 amino acids in length or shorter.

47.   The method of claim 40, wherein the reagant is detectably labeled.

5   48.   The method of claim 47, wherein the label is selected from the group consisting of a radioisotope, a bioluminescent compound, a chemiluminescent compound, a fluorescent compound, a metal chelate, or an enzyme.

49.   A method of treating an amyloid disorder associated with NAC comprising administering to a subject with the disorder, a therapeutically effective amount of reagent which modulates NACP activity.

50.   The method of claim 49, wherein the reagent is an antisense polynucleotide sequence.

51.   The method of claim 50, wherein the reagent is an antibody.

52.   The method of claim 51, wherein the antibody is monoclonal.

53.   The method of claim 49, wherein the reagant is a NAC peptide.

54.   The method of claim 53, wherein the NAC peptide is about 28 amino acids in length or shorter.

55.   The method of claim 49, wherein the reagant is detectably labeled.

56.   The method of claim 49, wherein the amyloid disorder is formation of neuritic plaques in the brain.

57.   The method of claim 49, wherein the reagent is a sense polynucleotide sequence.

58.   A method of treating an amyloid disorder associated with NAC comprising administering to a subject with the disorder, a therapeutically effective amount of reagent which modulates NAC aggregation.

59.   The method of claim 58, wherein the reagant is a NAC peptide.

60.   The method of claim 59, wherein the NAC peptide is about 28 amino acids in length or shorter.

61.   The method of claim 59, wherein the reagant is detectably labeled.

62.   The method of claim 58, wherein the amyloid disorder is formation of neuritic plaques in the brain.

63.   An isolated polynucleotide sequence which comprises 5' and 3' untranslated nucleotide sequences associated with the nucleotide sequence which encodes NACP.

64.   The polynucleotide of claim 63, wherein the polynucleotide is Sequence I.D. No 1.

65.   An isolated polynucleotide sequence which comprises nucleotide sequence encoding NAC polypeptide, or a fragment thereof.

66.   An isolated polypeptide wherein the polypeptide is encoded by the polynucleotide of Sequence ID. No. 1.

67.   The polypeptide of claim 66, wherein the polypeptide is Sequence I.D. No. 3.

68.   The polypeptide of claim 66, wherein the polypeptide is Sequence I.D. No. 4.

69.   The polypeptide of claim 66, wherein the polypeptide is Sequence I.D. No. 5.

70.   A transgenic mouse comprising a gene that encodes the polypeptide NACP, a fragment thereof, or a functional derivative thereof.

71.   A method for diagnosing AD by detecting the presence of NAC in amyloid in the brain tissue of a mammal, comprising:

5   (a)   administering a detectably labeled NAC peptide that will cross the blood/brain barrier of the mammal and bind any NAC therein into the blood circulation of the mammal; and,

            (b)   detecting any binding of NAC by the detectably labeled NAC peptide.

10   72.   The method according to claim 71 wherein the NAC peptide is about 28 amino acids in length or shorter.

73.   The method according to claim 71 wherein the NAC peptide is detectably labeled with a radioisotope.

74. The method according to claim 73 wherein any bound, detectably labeled peptide is detected by single photon emission computed tomography or postiron emission tomography.

75. The method according to claim 71 wherein the NAC peptide is detectably labeled with a paramagnetic isotope.

76. The method according to claim 75 wherein any bound, detectably labeled NAC peptide is detected by magnetic resonance imaging.

77. A method for evaluating the progression of amyloid formation in AD by detecting the presence of NAC in amyloid in the brain tissue of a mammal, comprising:

   (a)   administering a detectably labeled reagent that will cross the blood/brain barrier of the mammal and bind any NAC therein into the blood circulation of the mammal; and,

   (b)   detecting any binding of NAC by the detectably labeled reagent.

78. The method according to claim 77 wherein the reagent is a NAC peptide.

79. The method according to claim 78 wherein the NAC peptide is about 28 amino acids in length or shorter.

80. The method according to claim 77 wherein the reagent is an antibody.

81. The method according to claim 77 wherein the reagent is detectably labeled with a radioisotope.

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82.   The method according to claim 81 wherein any bound, detectably labeled reagent is detected by single photon emission computed tomography or postiron emission tomography.

83.   The method according to claim 77 wherein the reagent is detectably labeled with a paramagnetic isotope.

84.   The method according to claim 83 wherein any bound, detectably labeled reagent is detected by magnetic resonance imaging.

85.   A method for evaluating the progression of amyloid formation in AD by detecting the presence of NACP in amyloid in the brain tissue of a mammal, comprising:

      (a)   administering a detectably labeled reagent that will cross the blood/brain barrier of the mammal and bind any NACP therein into the blood circulation of the mammal; and,

      (b)   detecting any binding of NACP by the detectably labeled reagent.

86.   The method according to claim 85 wherein the reagent is a NACP peptide.

87.   The method according to claim 86 wherein the NACP peptide is about 28 amino acids in length or shorter.

88.   The method according to claim 85 wherein the reagent is an antibody.

89.   The method according to claim 85 wherein the reagent is detectably labeled with a radioisotope.

90.   The method according to claim 89 wherein any bound, detectably labeled reagent is detected by single photon emission computed tomography or postiron emission tomography.

60

91.   The method according to claim 85 wherein the reagent is detectably labeled with a paramagnetic isotope.

65   92.   The method according to claim 91 wherein any bound, detectably labeled reagent is detected by magnetic resonance imaging.
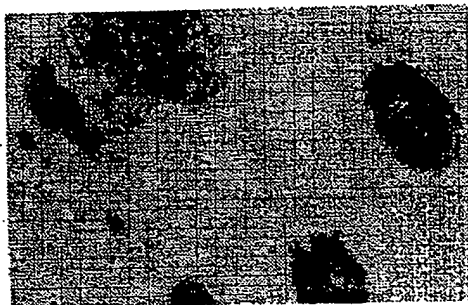
Fig. 1A
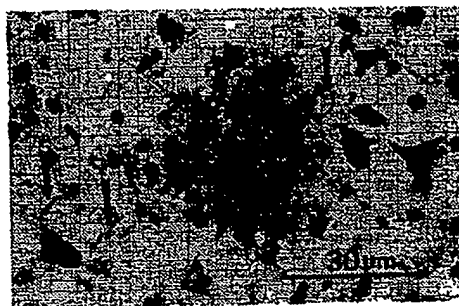
Fig. 1B
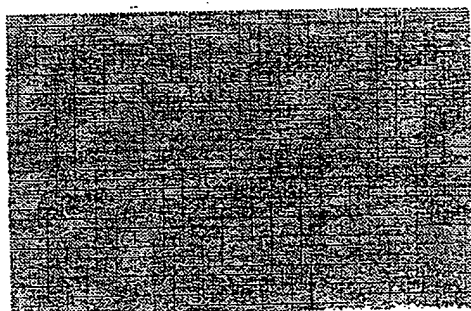
Fig. 1C

Fig. 1D

```
1    G CTC TCG GAG TGG CCA TTC GAC GAC AGT GTG GTG TAA AGG AAT TCA   46
                                                       *
47   TTA GCC ATG GAT GTA TTC ATG AAA GGA CTT TCA AAG GCC AAG GAG GGA   94
1         M   D   V   F   M   K   G   L   S   K   A   K   E   G      14

95   GTT GTG GCT GCT GAG AAA ACC AAA CAG GGT GTG GCA GAA GCA GCA      142
15    V   V   A   A   E   K   T   K   Q   G   V   A   E   A   A       30

143  GGA AAG ACA AAA GAG GGT GTT CTC TAT GTA GGC TCC AAA ACC AAG GAG  190
31    G   K   T   K   E   G   V   L   Y   V   G   S   K   T   K   E    46

191  GGA GTG GTG CAT GGT GTG GCA ACA GCT GAG AAG ACC AAA GAG CAA      238
47    G   V   H   G   V   A   T   A   E   K   T   K  [E   Q]           62

239  GTG ACA AAT GTT GGA GGA GCA GTG GTG ACG GGT GTG ACA GTA GCC      286
63    V   T   N   V   G   G   A   V   V   T   G   V   T   V   A        78
                                  X

287  CAG AAG ACA GTG GAG GGA GCA AGC ATT GCA GCC ACT GGC TTT          334
79   [Q   K]  T   V   E   G   A  [S   I]  A   A   T   G   F            94
                                    Y

335  GTC AAA AAG GAC CAG TTG GGC AAG AAT GAA GGA GCC CCA CAG GAA      382
95   [V]  K   K   D   Q   L   G   K   N   E   E   G   A   P   Q   E    110

383  GGA ATT CTG GAA GAT ATG CCT GTG GAT CCT GAC AAT GAG GCT TAT GAA  430
111   G   I   L   E   D   M   P   V   D   P   D   N   E   A   Y   E    126

431  ATG CCT TCT GAG GAA GGG TAT CAA GAC TAC GAA CCT GAA TAA GAA      478
127   M   P   S   E   E   G   Y   Q   D   Y   E   P   E   **           140
```

Fig. 2A-1

Fig. 2A-2

Fig. 2B

Fig. 3

```
            10
N---KAKEGV--
            21
  --KTKQGV--
            32
  --KTKEGV--
            43
  --KTKEGV--
            58
  --KTKEQV--
            80
  --KTVEGA--
           102
  --KNEEGA---C
```

Fig. 4A

```
            48
N---VVHGVATVA--
            70
  --VVTGVTAVA---C
```

Fig. 4B

```
              1         10        20        30        40          50
NACP   MDVFMKGLSKAKEGVVAAAEKTKQGVAEAAGKTKEGVLYVGSKT··KEGVVHGVATV
       :::: :: : :::::::: ::::::::: ::: ::::::::       ::
EST    MDVFKKGFSIAKEGVVGAVEKTKQGVTEAAEKTKEGVMYVGLHFFFKERIN*······
              1         10        20        30        40          50
```

Fig. 4C

Fig. 5A

28S →

18S →
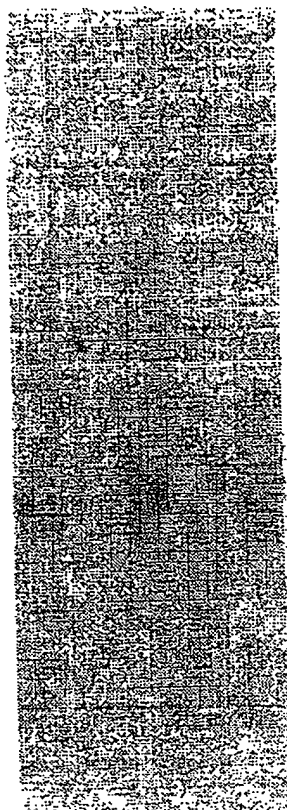
Fig. 5B

1   2   3   4   5

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Fig. 6A

Fig. 6B

DAY

0 1 2 3 5 7

GEL TOP

k Da

46
30

21.5

14.3

6.5

3.4

2.35
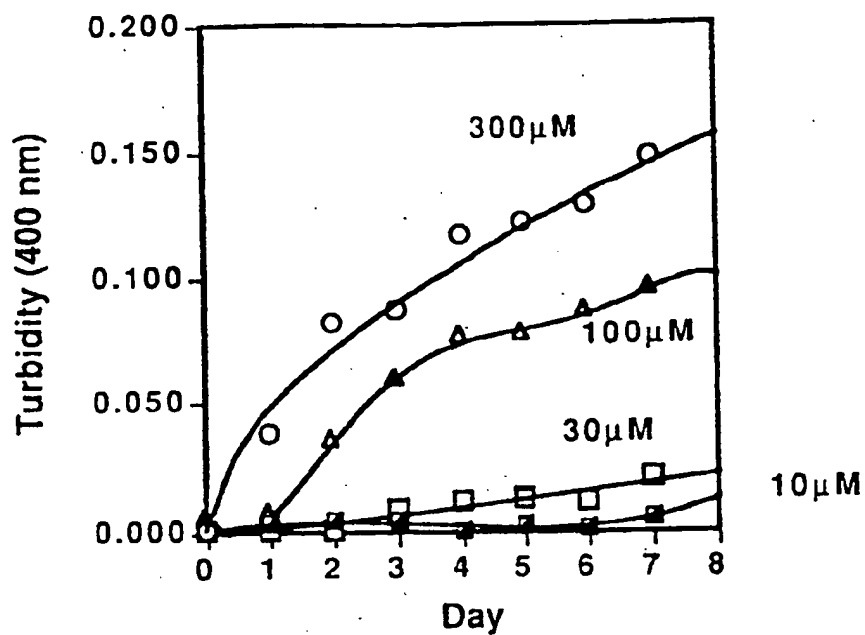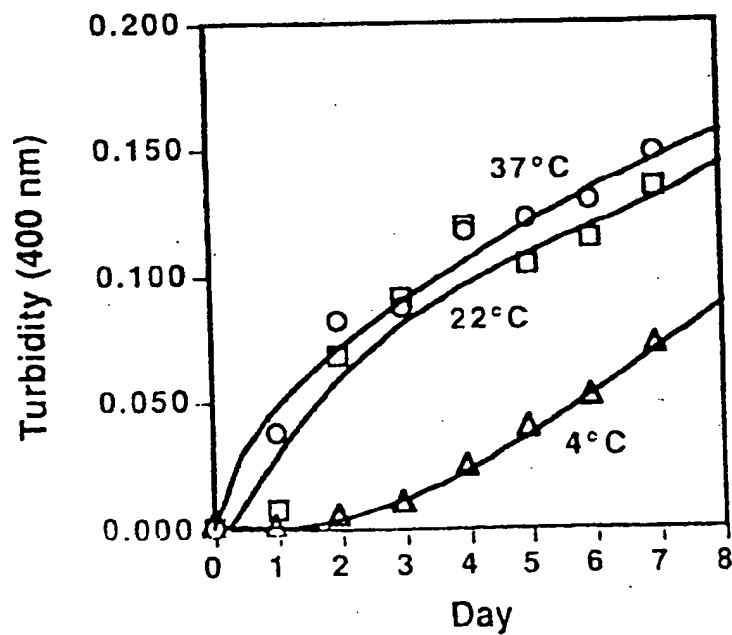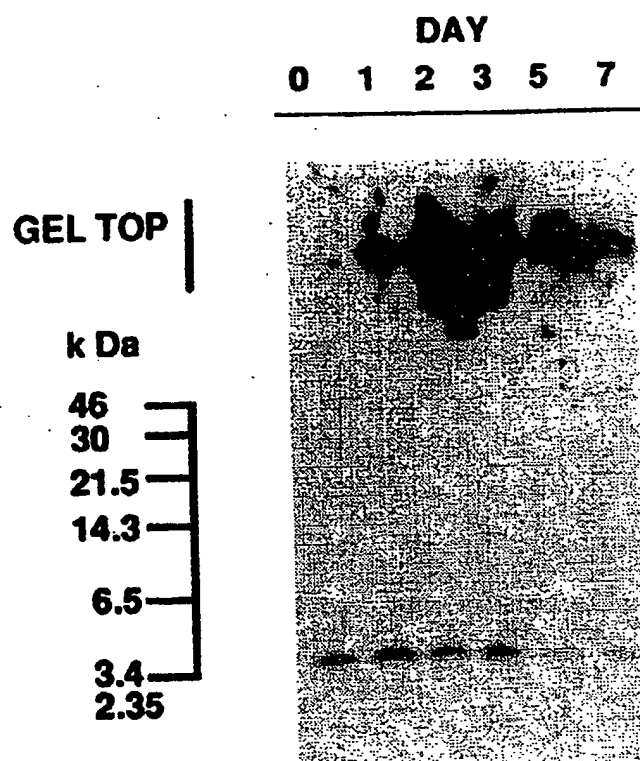
Fig. 7

Fig. 8A

Fig. 8B
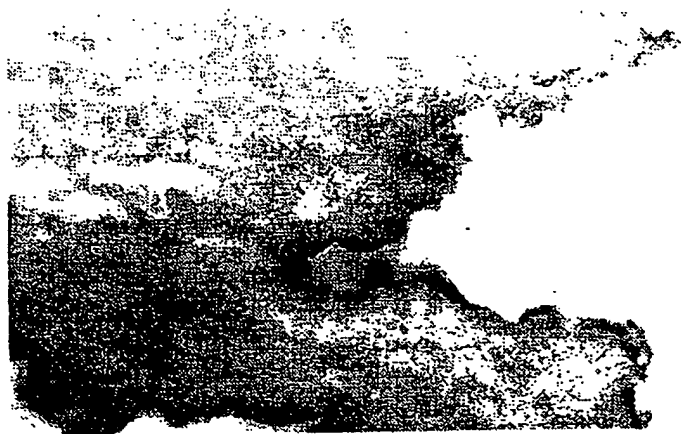
Fig. 9A
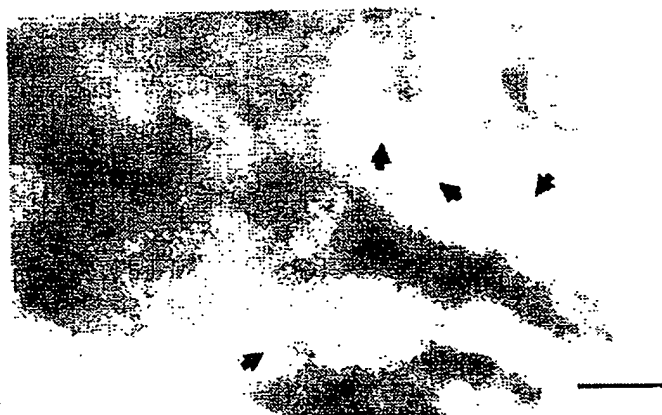
Fig. 9B
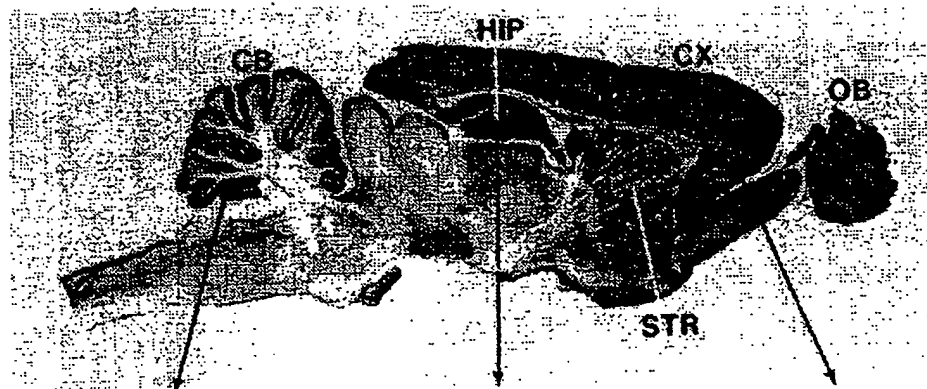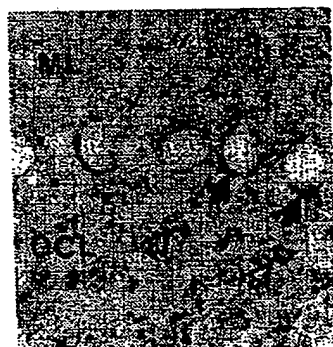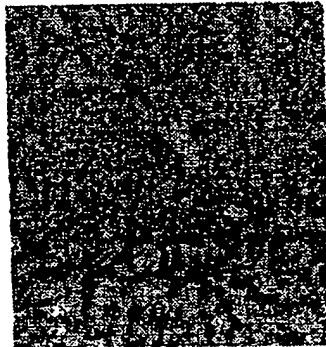
Fig. 10A

Fig. 10B   Fig. 10C   Fig. 10D
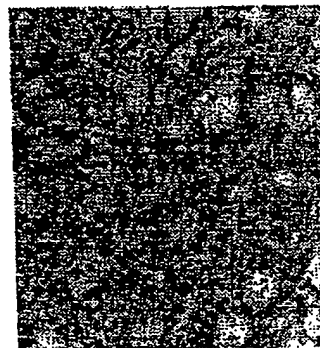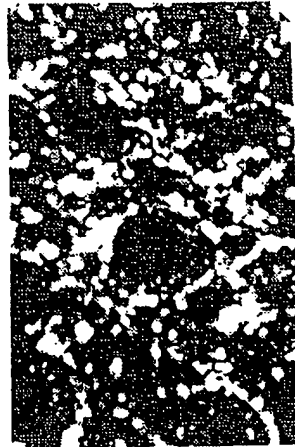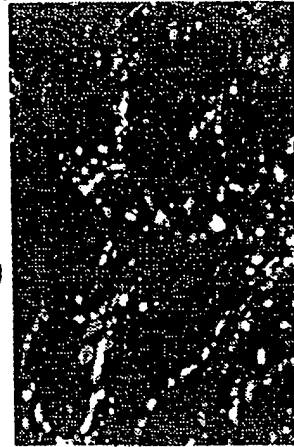
Fig. 11C

Fig. 11F

Fig. 11I

Fig. 11B
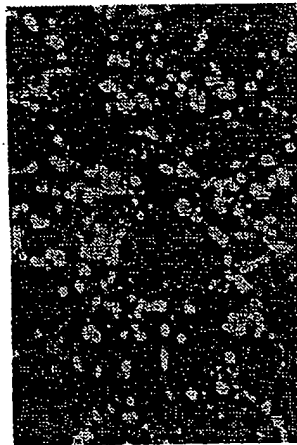
Fig. 11E
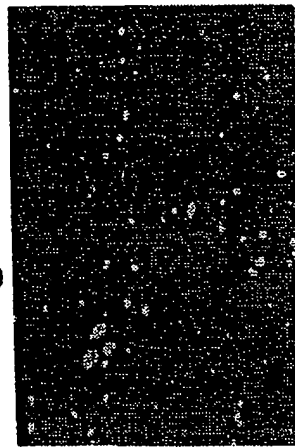
Fig. 11H
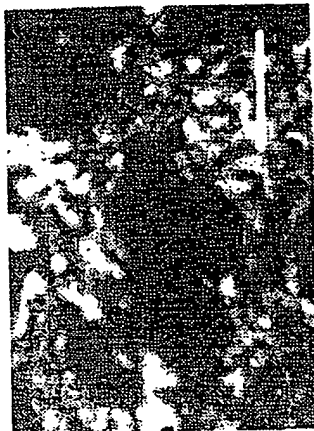
Fig. 11A
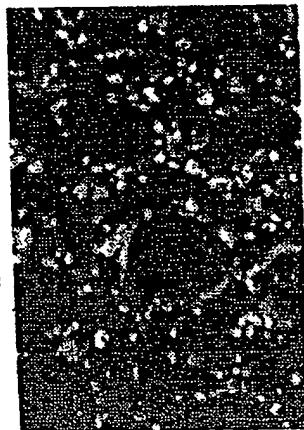
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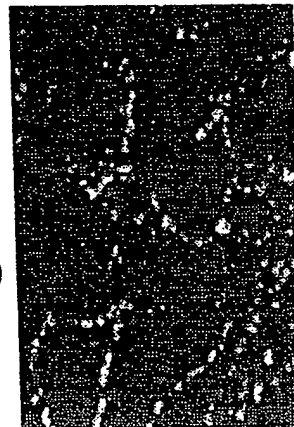
Fig. 11G
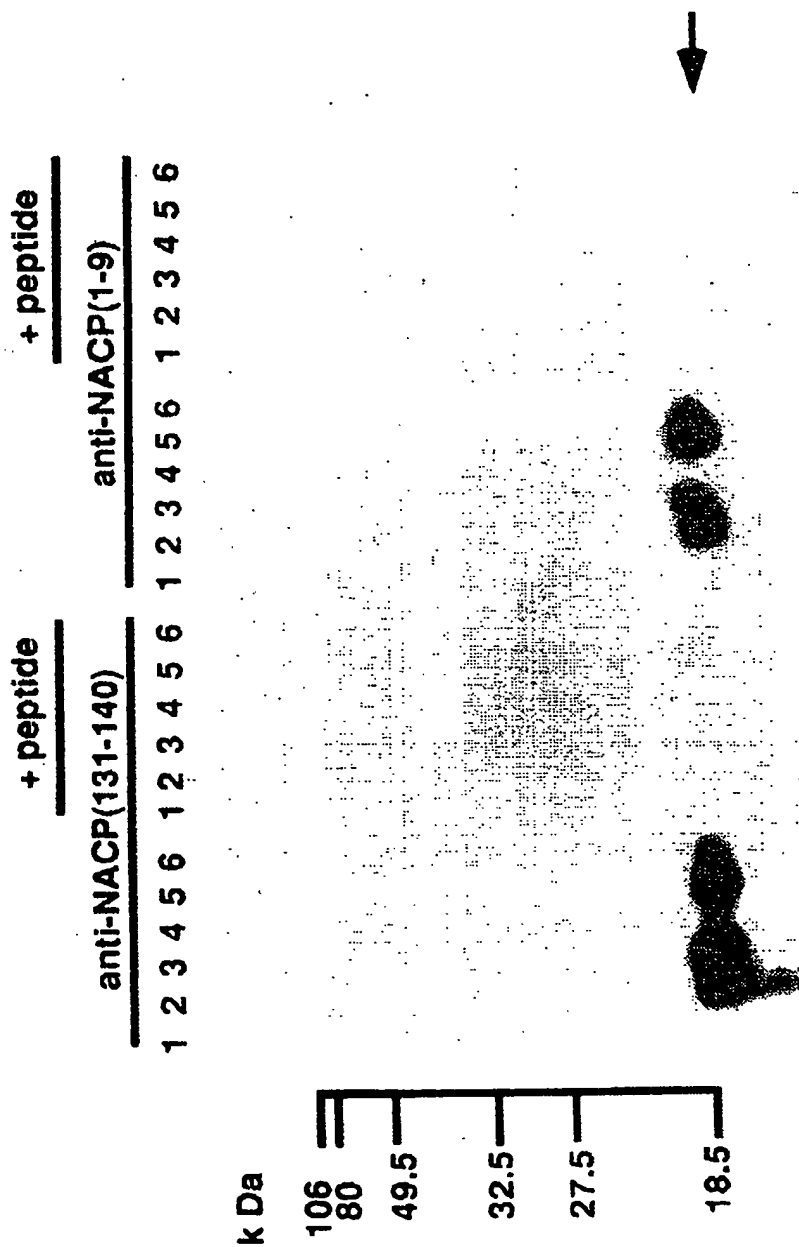
Fig. 14
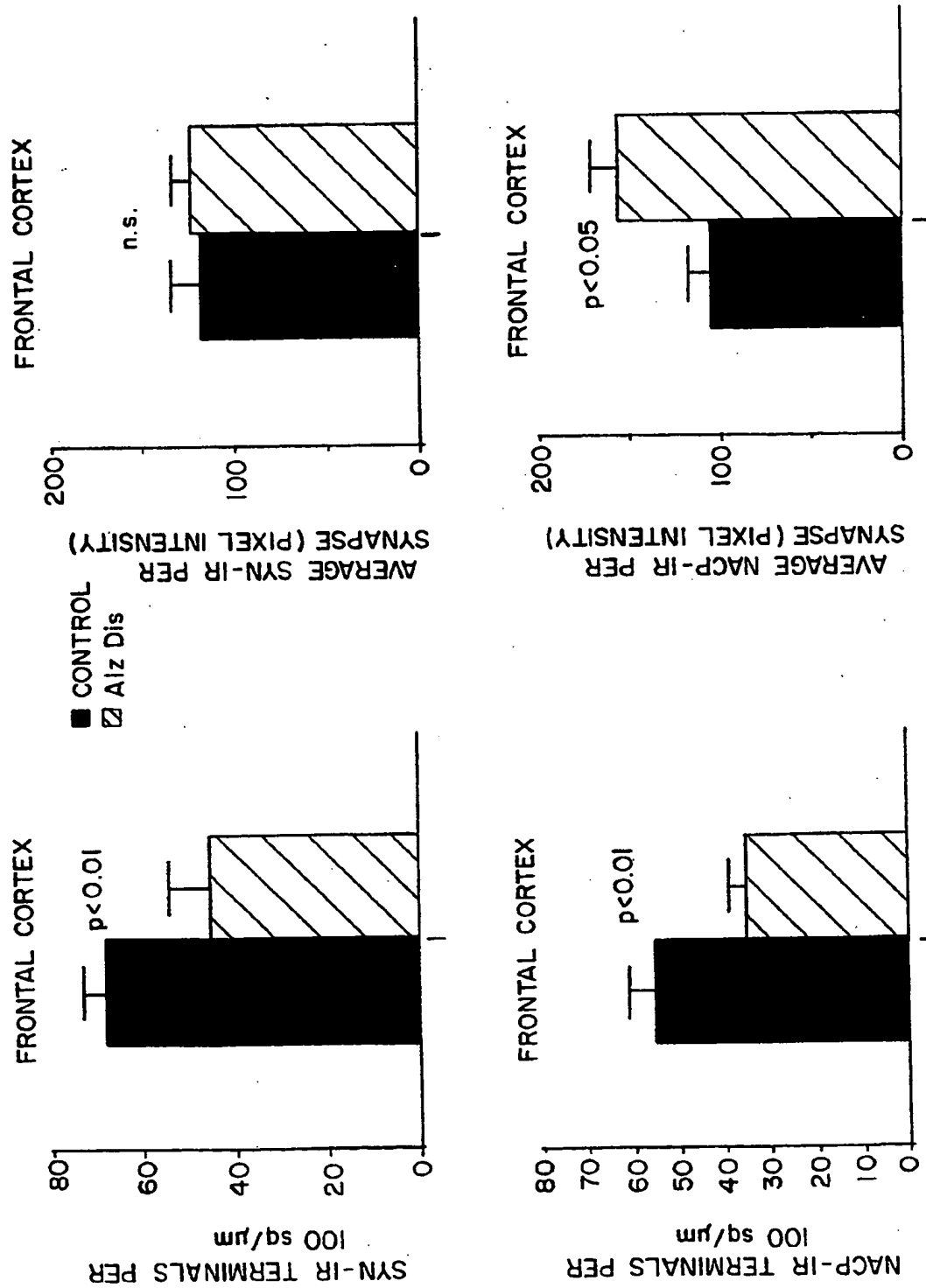
FIG. 15
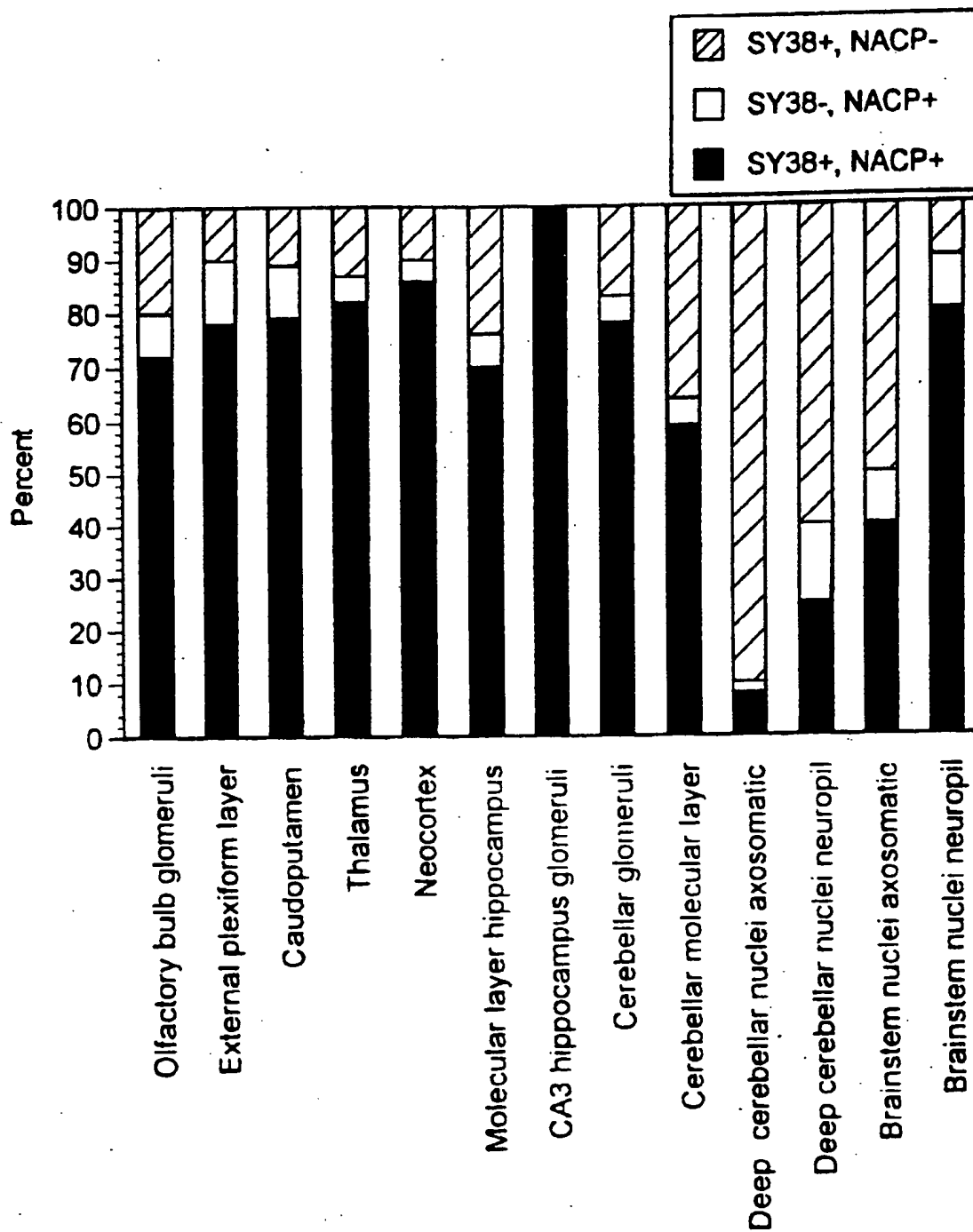
Fig. 12

Fig. 13

CONTROL

NACP SYNAPTOPHYSIN
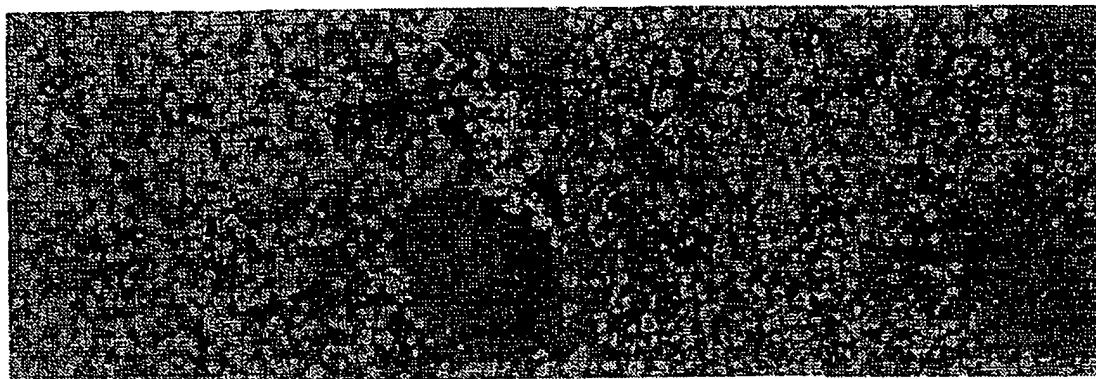
ALZHEIMER DISEASE
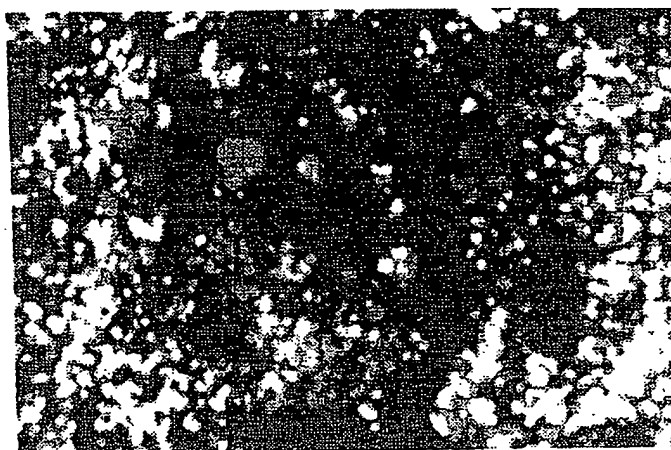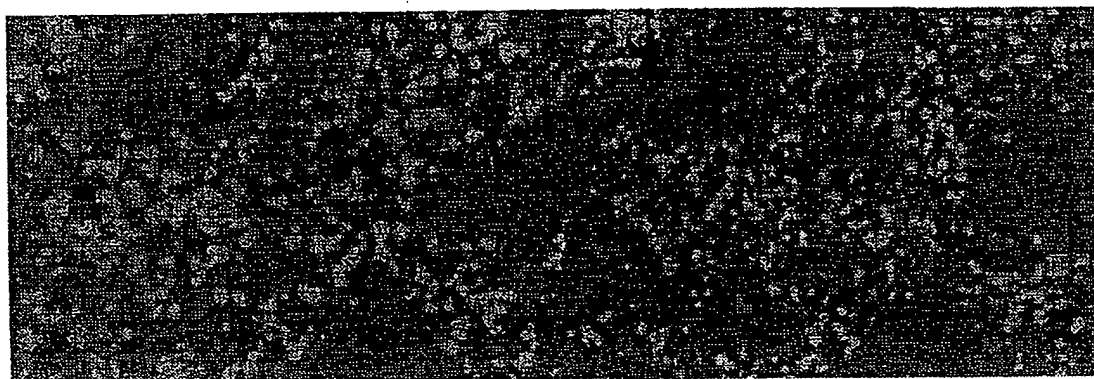
Fig. 16

SUBSTITUTE SHEET (RULE 26)

Anti-NAC (XI)   Anti-amyfold (4G8)
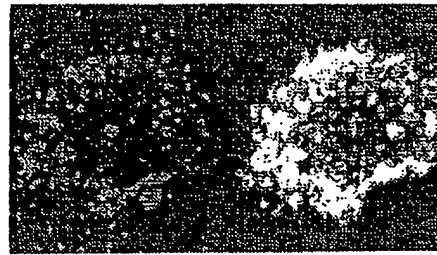
Fig. 17A

Fig. 17B
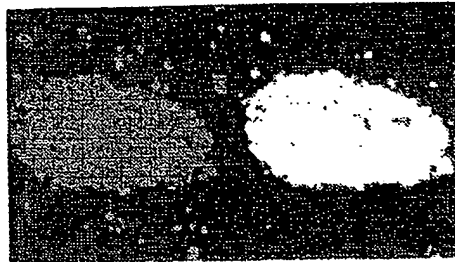
Fig. 17C
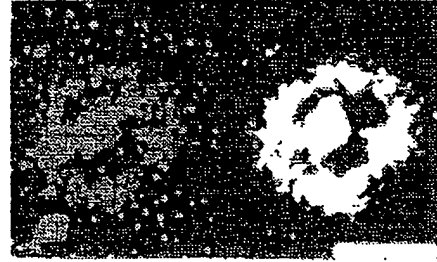
Fig. 17D
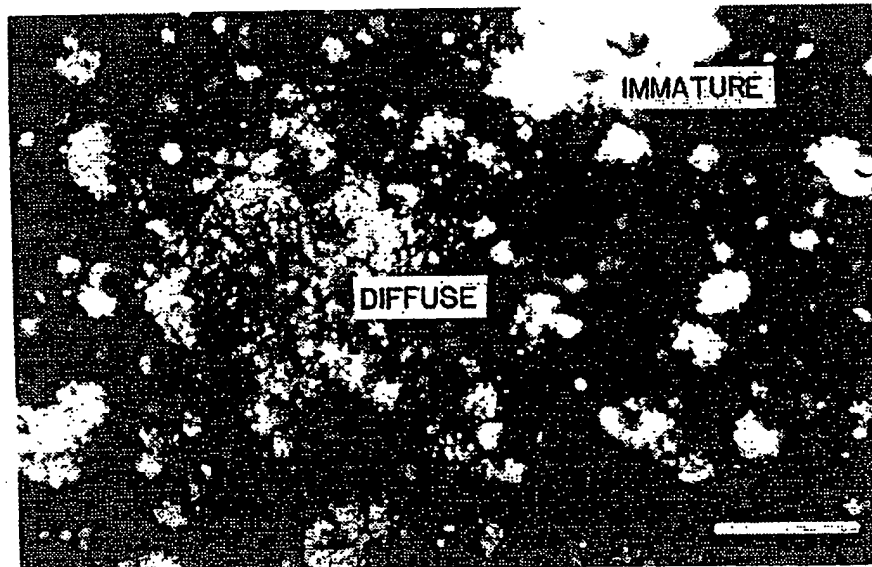
Fig. 17E

21/21
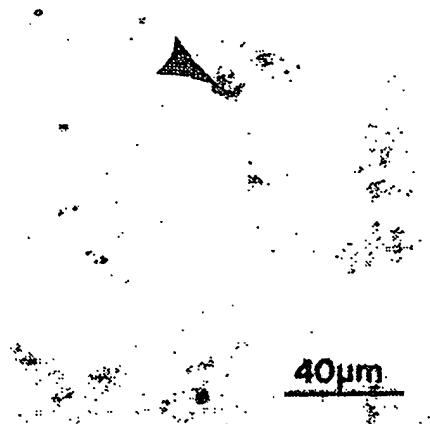
Fig. 18A

Fig. 18B
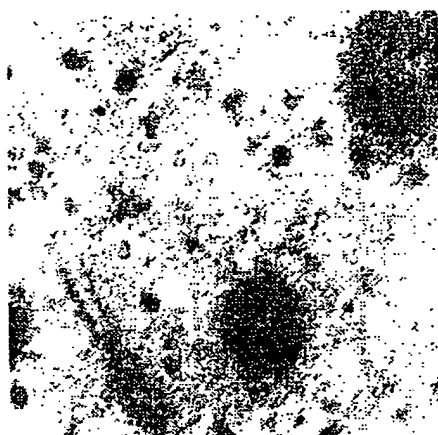
Fig. 18C
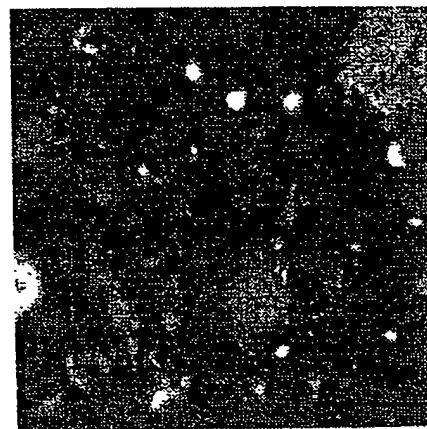
Fig. 18D

Fig. 18E
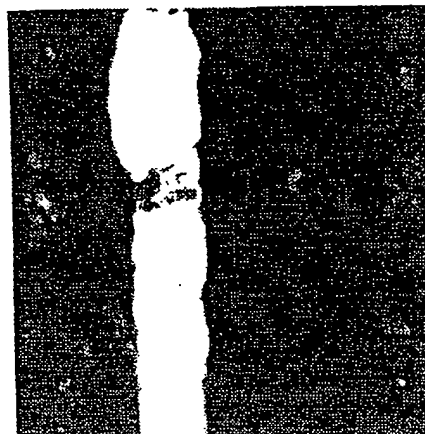
Fig. 18F

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 7.1, 7.2, 7.8, 69.1, 240.2, 252.3, 320.1; 514/2, 12; 530/300, 326, 387.1; 536/22.1, 23.1, 23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

None

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Biosis, CAS Online, APS

Search terms: alzheimer?, novel amyloid component?, amyloid fibril#, prea4, protein x, protein y

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PROC. NATL. ACAD. SCI. USA, Vol. 82, issued June 1985, Masters et al., "Amyloid plaque core protein in Alzheimer disease and Down syndrome", pages 4245-4249, see entire document.	1-92
A	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, Vol. 120, No. 3, Issued 16 May 1984, Glenner et al., "Alzheimer's Disease: Initial Report of the Purification and Characterization of a Novel Cerebrovascular Amyloid Protein", pages 885-890, see entire document.	1-92

[X] Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	
"E"	earlier document published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means	
"P"	document published prior to the international filing date but later than the priority date claimed	"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
09 DECEMBER 1994	**19 DEC 1994**

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Hyosuk Kim Jill Warden for
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	AMERICAN JOURNAL OF PATHOLOGY, Vol. 141, No. 4, issued October 1992, Price et al., "Amyloidosis in Aging and Alzheimer's Disease", pages 767-772, see entire document.	1-92
X, P	PROC. NATL. ACAD. SCI. USA, Vol. 90, issued December 1993, Ueda et al., "Molecular cloning of cDNA encoding an unrecognized component of amyloid in Alzheimer disease", pages 11282-11286, see entire document.	1-92

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A01N 37/18; A61K 35/14, 38/00; C07H 17/00, 19/00, 21/00; C07K 1/00, 2/00, 4/00, 5/00, 7/00, 14/00, 16/00, 17/00; C12N 1/20, 5/00, 15/00; C12P 21/00; C12Q 1/00, 1/68

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/6, 7.1, 7.2, 7.8, 69.1, 240.2, 252.3, 320.1; 514/2, 12; 530/300, 326, 387.1; 536/22.1, 23.1, 23.5